BIOCHEMICAL, PHYSIOLOGICAL, AND MOLECULAR CHARACTERIZATION OF MANNITOL ACCUMULATING TRANSGENIC WHEAT IN RESPONSE TO WATER DEFICIT STRESS

By

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION 1

II. REVIEW OF LITERATURE 5

Water stress physiology 6
Water relations 6
Osmotic adjustment 7
Solutes contributing to OA 8
Photosynthesis and other growth parameters under water stress 10
Mannitol in higher plants 11
Transgenic approach: pathway for a second green revolution 13
Oxidative stress 15
Generation of ROS 15
Role of ROS 17
ROS scavenging mechanisms in plants 18
Role of sugar alcohols in ROS scavenging 20
Molecular characterization of mtlD gene 22
Estimation of transgene copy number 22
Quantification of transgene expression 24
Objectives 25

III. METHODOLOGY 27

Plant Materials 28
Polymerase chain reaction (PCR) 28
Southern blot analysis 29
Objective 1 30
Water potential parameters 31
Osmotic adjustment 32
Relative water content 33
Gas exchange measurements 33
Chlorophyll fluorescence 34
Phenotype measurements 35
Objective 2 35

Objective 3 36
Lipid peroxidation assay 36
Chemiluminescene test for hydroxyl radicals 37
Objective 4 38
Determination of transgene copy number 38
Transgene expression profiling 41
Statistical analysis 43

IV. RESULTS 44

Water stress imposition 45
PCR screening for the transgene 45
Southern analysis 45
Growth parameters 45
Water relations 46
Gas exchange parameters 46
Chlorophyll fluorescence 47
Lipid peroxidation 47
Antioxidant capacity of leaf extracts 47
Carbohydrate analysis 48
Transgene expression 48
Transgene copy number 49

V. DISCUSSION 50

REFERENCES 59

LIST OF TABLES

4.1: Fresh weight and dry weight of the above ground biomass in T_4 at 30 DAS.....78

4.2: Plant height and number of tillers in T_4 at 30 DAS 78

4.3: Relative water content (%) of leaf tissue in T_3. Data measured on the mid section of the same leaf from which Ψw data were obtained.....79

4.4: Relative water content (%) of leaf tissuein T_4. Data measured on the mid section of the same leaf from which Ψw data were obtained 79

4.5: Water potentials (Ψw, MPa) and osmotic adjustment (OA, MPa) in T_3. Data measured from basal part of the youngest leaf 80

4.6: Water potentials (Ψw, MPa) and osmotic adjustment (OA, MPa) in T_4. Data measured from basal part of the youngest leaf 81

4.7: Chlorophyll fluorescence (Fv/Fm) measured on the flag leaf after 30 min of dark adoption in T_3.....82

4.8: Chlorophyll fluorescence (Fv/Fm) measured on the flag leaf after 30 min of dark adoption in T_4 83

4.9: Malondialdehyde content of the leaf tissue in T_3 at 30 DAS (nmol g^{-1} fwt).....84

4.10: Malondialdehyde content of the leaf tissue in T_4 at 30 DAS (nmol g^{-1} fwt).....84

4.11: Antioxidant capacity of leaf extracts in T_3 at 30 DAS (% inhibition of pholasin luminescence).....85

4.12: Antioxidant capacity of leaf extracts in T_4 at 30 DAS (% inhibition of pholasin luminescence).....85

4.13: Carbohydrate concentration in leaf tissue in T_3 at 15 DAS (µmol g^{-1} fwt).....86

4.14: Carbohydrate concentration in leaf tissue in T_4 at 15 DAS (µmol g^{-1} fwt) 86

4.15: Carbohydrate concentration in leaf tissue in T_3 at 30 DAS (µmol g^{-1} fwt) 87

4.16: Carbohydrate concentration in leaf tissue in T_4 at 30 DAS (µmol g^{-1} fwt) 87

LIST OF FIGURES

4.1: Water content in the experimental pots measured using Time Domain Reflectometry (Bars are ±SE, n=4).....88

4.2: PCR screening of TA2-118 transgenic wheat line for presence of the *mtlD* (600 bp) and *bar* (300 bp) genes.....89

4.3: Southern blot analysis for the *mtlD* gene in T_3. Genomic DNA from four transgenic lines, TA2-110, TA2-118, TA5-104, and TA5-108 were hybridized with a 600 bp radiolabelled probe.....89

4.4: Net photostnthesis rate following imposition of water stress in T_3 (Bars are ±SE, n=4) 90

4.5: Net photosynthesis rate following imposition of water stress in T_4 (Bars are ±SE, n=4).....91

4.6: Transpiration rate following imposition of water stress in T_3 (Bars are ±SE, n=4) 92

4.7: Transpiration rate following imposition of water stress in T_4 (Bars are ±SE, n=4) 93

4.8: Stomatal conductance following imposition of water stress in T_3 (Bars are ±SE, n=4) 94

4.9: Stomatal conductance following imposition of water stress in T_4 (Bars are ±SE, n=4) 95

4.10: Relative expression levels of the *mtlD* gene in T_3 (2-118s at 15 DAS used as calibrator) 96

4.11: Relative expression levels of the *mtlD* gene in T_4 (2-118s at 15 DAS used as calibrator) 97

4.12: Number of *mtlD* copies per haploid wheat genome 98

LIST OF ABBREVIATIONS

Ψs	Solute potential
Ψw	Water potential
APX	Ascorbate peroxidase
bp	Base pair
CAT	Catalase
Ct	Threshold cycle
cv	Cultivar
DAS	Days after stress
DNA	Deoxyribonucleic acid
dNTP's	Deoxynucleotide triphosphate
DMSO	Dimethyl sulfoxide
fwt	fresh weight
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX	Glutathione peroxidase
GSH	Reduced form of glutathione
H_2O_2	Hydrogen peroxide
HPAE	High performance anion exchange
M1P	Mannitol 1-phosphate
MDA	Malondialdehyde
MPa	Mega pascals
mtlD	Mannitol 1-phosphate dehydrogenase
MTD	Mannitol dehydrogenase
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
OA	Osmotic adjustment
PAD	Pulsed amperometric detection
pAHC20	Wheat transformed with bar gene construct alone
PCR	Polymerase chain reaction
PRK	Phosphoribulokinase
PS I	Photosystem I
PS II	Photosystem II
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
Rubisco	Ribulose- 1,5-bisphosphate carboxylase/oxygenase
RUBP	Ribulose-1,5-bisphosphate
RWC	Relative water content

SOD	Superoxide dismutase
TA2	Transfomed wheat with mannitol biosynthesis targeted into cytosol
TA5	Transformed wheat with mannitol biosynthesis targeted into chloroplasts
Taq	*Thermus aquaticus*
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TDR	Time domain reflectometry
Ubi-1	Ubiquitin-1
VWC	Volumetric water content

CHAPTER I

INTRODUCTION

Abiotic stresses pose the greatest challenge to scientists and farmers alike in their efforts to improve agricultural productivity. Increasing human population coupled with demand for shrinking resources has fueled interest in the scientific community to elucidate the mechanisms by which plants respond to stress and in manipulating these mechanisms to increase plant productivity under adverse conditions. Analysis of major crops in the United States has shown that there is a large genetic potential for yield that is unrealized (Boyer, 1982) and drought alone accounts for 25% of the loss in crop yields (Kramer and Boyer, 1995). In 2003, drought related crop insurance indemnity payments accounted for 45% of total crop insurance payments. Since 1989 the Federal Crop Insurance Corporation (FCIC) has paid on an average US $500 million for drought related losses annually (http://www.usda.gov/Newsroom/fs0199.04.html).

Wheat (*Triticum aestivum* L.) is one of the World's most important cereal crops, contributing one fifth of the World's total food calories (Zohary and Hopf, 2000). Wheat production is severely limited by environmental stress. Wheat production in the United States showed a continuous downward trend for five years preceding 2002 hitting an all time low harvest of 43.9 MT in 2002 (FAO Stat Databases, 2004). One of the major abiotic factors contributing to this alarming scenario of decreasing wheat yields is water-deficit stress. Water is indispensable to maximize crop production and shortage of water has serious consequences. Water deficit at various degrees may lead to temporary reduction in growth rate, reduced yields, or even permanent wilting and death by dehydration. Therefore, in order to survive as well as thrive under water-deficit conditions, plants have to develop mechanisms to cope with this stress. Water deficit

elicits complex responses that are manifested in changes at cellular, physiological and developmental levels (Bray, 1993).

Breeding for tolerance in crop plants has given some very good results but involves some failures as well. This is mainly because the response to water deficit is a very complex phenomenon that is affected by several genes. Recently, scientific efforts to improve plant productivity have focused on reducing the gap between farm yields and the genetic potentials for yield by modifying physiological processes (Boyer, 1982). One of the most promising approaches is to engineer plants with novel genes and induce new biosynthetic pathways hitherto not present in those plants to improve their tolerance to stress. Several studies suggested and demonstrated that plants genetically engineered for the accumulation of compatible solutes hold promise of increased drought tolerance (Holmberg and Bulow, 1998; Bajaj et al., 1999; Rathinasabapathi, 2000). Some of the compatible solutes studied so far include amino acids, tertiary sulfonium compounds, quaternary ammonium compounds and polyhydric sugar alcohols (Pilon-Smits et al., 1995; Shen et al., 1997; Rontein et al., 2002; Chen and Murata, 2002). In tune with the above views, a bacterial *mtlD* gene encoding for mannitol-1-phosphate dehydrogenase was transformed into spring wheat (*cv*. Bobwhite) for accumulation of mannitol (Abebe et al., 2003). Two gene constructs were used during transformation to induce mannitol biosynthesis in the cytosol or the chloroplasts in different transgenic events. A negative control was generated with a construct containing only the selectable-marker *bar* gene.

Mannitol, a sugar alcohol is known to be involved in osmotic adjustment (OA) and osmoprotection in plants like celery in which it is naturally produced (Zamski et al., 2001). Members of the polyol family, to which mannitol belongs, are known to minimize the reduction in cell turgor under water stress when they accumulate to high levels (Popp and Smirnoff, 1995). Bray (1997) suggested that the improved stress tolerance in transgenic plants accumulating mannitol might be due to maintenance of water potential gradients at the whole plant level even in the absence of OA.

Mannitol is known to be a free radical scavenger, specifically of hydroxyl radicals (Shen et al., 1997). Reactive oxygen species (ROS) accumulate rapidly during stress and have to be detoxified instantaneously to reduce cell membrane and organelle damage (Grassmann et al., 2002).

Several studies have reported an unpredictable behavior in transgene inheritance and expression. Bourdon et al. (2002) reported that initially high levels of transgene expression was not maintained in subsequent generations, and Iyer et al. (2000) suggested the occurrence of transgene silencing due to high copy number of transgenes present in the genome. Previous experiments conducted by Abebe et al. (2003) confirmed the integration and expression of the *mtlD* gene in the wheat genome of T_0 transformants. In this study we used real-time PCR to determine the transgene copy number and its expression in the T_3 and T_4 generations.

CHAPTER II

REVIEW OF LITERATURE

Water Stress Physiology

Availability of adequate water during the season is very important to attain maximum crop productivity, because much of the resource exchanges that occur between the plant and its environment occur through this medium. Low precipitation, low water holding capacity of the soil, salinity, and low/ high temperatures or a combination of these factors can limit water availability to plants. The maintenance of turgor is necessary to allow many metabolic processes to function normally and contribute to growth. The study of water potential and its components provides us with information about the water status of the plant.

Water Relations:

Water contained in plants is divided into two components: apoplastic and symplastic. The movement of water in the plant and across these two compartments depends upon the water potential. Under normal conditions the water potential of the cell and its surroundings will create a gradient along which water moves from high to low potential. When the plants are exposed to water stress due to high transpiration losses or low water uptake by roots, the cells start losing water leading to loss of turgor. As a result of the volume reduction cells concentrate solutes, thereby lowering the osmotic potential. Accumulation of new solutes tends to minimize volume loss and turgor reduction (Kramer and Boyer, 1995).

Four main classes of osmotically active solutes can affect the osmotic potentials in the cells. Simple carbohydrates such as sugars (sucrose, glucose, fructose), sugar alcohols

(sorbitol, mannitol), and amino acids (proline and glycine-betaine), known as compatible solutes, accumulate mainly in the cytosol. Inorganic ions (K^+, Na^+, Ca^+, Cl^-, NO_3^-, and HPO^{4-}) and organic acids (citric acid and malic acid in CAM plants) accumulate primarily in the vacuole. All of these lower the osmotic potential of the cell.

Resistance of some plants to water-deficit stress derives from the ability to maintain high turgor potential during conditions of stress in the environment. Many drought tolerant plants reduce the osmotic potential, and as a consequence water potential. This guards against water loss and thus minimizes the loss of turgor during stress. The response is called osmotic adjustment and is discussed in greater detail below.

Osmotic Adjustment:

Osmotic adjustment (OA) in higher plants refers to the lowering of osmotic potential arising from the net accumulation of solutes in response to water deficits or salinity. OA occurs in leaves, hypocotyls, roots, and reproductive organs. The degree of adjustment is influenced by factors such as the rate of development of water stress, degree of water deficit, genotypes and environmental conditions (Turner and Jones, 1980). OA, unlike stomatal closure and reduction in leaf area, provides the potential for maintaining photosynthesis and growth under increasing water deficit stress. OA has contributed to stabilization of grain yields in wheat (Moinuddin et al., 2005) and varieties with greater capacity to adjust osmotically have performed better in the field (Morgan et al., 1986).

OA in response to water deficit is considered a beneficial drought tolerance mechanism in several crops (Morgan, 1984) including wheat (Johnson et al., 1984). Flagella et al.

(1996) reported that OA arises only under severe stress and is of greatest degree when the rate of stress development is low. Furthermore, it is known that the extent of OA is also dependent on the developmental stage and environmental factors (Johnson et al., 1984). Shangguan et al. (1999) reported that a high degree of OA was observed in wheat when drought stress was applied in early stages and at a low drought development rate.

Solutes contributing to OA:

Major contribution to OA comes from the accumulation of inorganic ions in the vacuole. K^+ and Cl^- are the two ions that contribute the most to OA (Hu and Schmidhalter, 1998). The source of inorganic ions is from the external medium and is more energy efficient for the cell compared to the synthesis of organic solutes (Yeo, 1983). But high concentrations of inorganic ions can lead to ion imbalance, nutrient ion deficiency, and ion toxicity disrupting normal metabolism in the cell when the stress periods are prolonged (Nabil and Coudret, 1995). An alternative but energy dependent way available to the plant is to accumulate organic solutes (osmolytes) like sugars, sugar alcohols, amino acids, and organic acids in the cell (Greenway and Munns, 1980). Osmolytes are compatible organic solutes that can accumulate in the cell without disrupting metabolic functions (Bartels and Nelson, 1994). Some of the osmolytes accumulating in the cells due to stress include amino acids (proline), sugar alcohols (mannitol), soluble sugars (fructans) and quaternary ammonium compounds (glycine-betaine). Accumulation of osmolytes occurs through *de novo* synthesis or through a combination of synthesis and catabolism.

The selective advantage of organic osmolytes over inorganic ions is due to their compatibility with macromolecular structure and function even at high concentrations. Osmolyte compatibility is proposed to result from the absence of osmolyte interaction with substrates and cofactors, and the non-perturbing effects of macromolecular interactions (Yancey et al., 1982). Sugars are known to be the major contributors of OA among osmolytes. Both reducing sugars (Kameli and Losel, 1995) and non-reducing sugars (Johnson et al., 1984) have been reported to play a major part in OA of wheat under water stress. Compatible organic solutes increase tolerance to water deficit stress through several mechanisms; osmotic adjustment, reactive oxygen species (ROS) scavenging, acting as a sink for reducing power thereby lowering the rate of ROS production, and storage of carbon and nitrogen.

Polyols with their OH groups can effectively replace water in establishing hydrogen bonds in case of limited water availability and therefore protect enzyme activities and membrane structures (Popp and Smirnoff, 1995). Mannitol, a sugar alcohol is known to be involved in osmotic adjustment and osmoprotection in celery among other plants. Mannitol and glycerol are common cell osmolytes in salt tolerant plants and aid in cell water retention also when exposed to freezing conditions (Yancey et al., 1982). Mannitol accumulation increases with an increase in salinity in celery, which showed substantial tolerance to increased levels of NaCl (Everard et al., 1994). Modest increases in levels of fructans (Pilon-Smits et al., 1995), trehalose (Garg et al., 2002), proline (Hare and Cress, 1997) and glycine-betaine occurred in plants that showed increased tolerance to stress, but the solute levels appeared too low to contribute substantially to OA.

Photosynthesis and other growth parameters under water stress:

All aspects of plant growth and metabolism are affected by water stress at all stages of growth. Water stress severely reduces net photosynthesis in flag leaf, top internode and ear of wheat (Wardlaw, 1971). The photosynthetic responses to water stress are both stomatal and non-stomatal. The initial response of the plant to drought is stomatal closure, and later inhibition of chloroplast activity, both eventually leading to reduced photosynthesis (Matthews and Boyer, 1984). Closed stomata and inhibition of chloroplast activity at low leaf water potentials decrease the leaf capacity to fix available CO_2 (Boese et al., 1997). Chloroplast inhibition cannot be overcome by an increase in the concentration of CO_2. Along with net photosynthesis, the transpiration rate also shows a reduction due to closure of stomata. The existence of a vapor pressure gradient from leaf to air commonly keeps the transpiration going even though at smaller rates when the photosynthesis is severely inhibited (Morant- Manceau et al., 2004).

Reduced intracellular CO_2 concentration (Ci) due to closed stomata leads to increased photorespiration. When coupled with high light conditions, the potential free radical formation and photoinhibition increases greatly in the chloroplast (Bjorkman and Powles, 1984). The capacity of carbon assimilation and utilization is reduced due to reduced activity of the enzymes involved such as rubisco (Parry et al., 2002), phosphoribulo kinase, and fructose-1, 6- bisphosphatase (Haupt-Hertig and Fock, 2002). Water stress causes changes in the pH in the chloroplast. Acidification of stroma and reduction in ATP synthesis are thought to cause enzyme inactivity and reduced RuBP turnover (Tezara et al., 1999). Increase in the rate of degradation of chlorophyll and the resultant decrease in

chlorophyll concentrations have been observed under prolonged water deficit stress (Bjorkman and Powles, 1984). Chlorophyll fluorescence is used as an indicator of stress in plants. However the electron transport chain is rather resistant to water deficit stress (Cornic and Massacci, 1996). Use of fluorescence data with gas exchange measurements can provide us with a good understanding of the stress response of the plant.

Water stress leads to reduced apical growth and spikelet formation, lower biomass accumulation (Wardlaw, 1971) and suppress tillering in wheat (Keim and Kronstad, 1981) ultimately resulting in loss of grain yield. Grain yield loss in wheat during water stress is also due to smaller number of grains per spike resulting from male sterility (Saini and Aspinall, 1981).

Mannitol in Higher Plants:

Sugar alcohols (acyclic polyols or alditols) are widely distributed in higher plants with mannitol being the most common member found in over 70 families (Lewis and Smith, 1967). Celery (*Apium graveolens L.*) has been used as a model system to study mannitol metabolism in higher plants. Pulse chase experiments in celery have shown that mannitol and sucrose are two major products of photosynthesis produced in roughly equal quantities (Loescher et al., 1992). In celery mannitol accounts for 10 to 60% of the carbon exported from mature leaves (Daie, 1986), and it is the predominant substrate translocated in the dark when sucrose pools are low (Davis and Loescher, 1990). The proposed mannitol biosynthesis pathway in higher plants is given below:

Fructose 6-phosphate -→ Mannose 6-phosphate-→ Mannitol 1-phosphate-→ Mannitol

A NADPH dependant mannose-6 phosphate reductase, mannose 6-phosphate isomerase, and mannitol 1-phosphate phosphatase are involved in the process (Loescher et al., 1992). All the enzymes involved in mannitol biosynthesis in plants are localized in the cytosol (Rumpho et al., 1983).

The above pathway differs from the mannitol biosynthesis of lower organisms like bacteria and brown algae (Ikawa et al., 1972):

Fructose 6-phosphate -→ Mannitol 1-phosphate -→ Mannitol

A NAD dependant mannitol 1-phosphate dehydrogenase (*mtlD* and mannitol 1 - phosphate phosphatase are the enzymes catalyzing the biosynthesis. The proposed pathway in the transgenic wheat lines under study is similar to the latter pathway. Mannitol biosynthesis in plants occurs simultaneously with other sugars and so far no plant has been reported to exclusively synthesize mannitol (Stoop et al., 1996).

Mannitol is translocated from source to sink organs in higher plants. The presence of mannitol in phloem sap and detection of mannitol in the sink organs where it is not synthesized provides support for long distance transport (Loescher, 1987; Moing et al., 1992). Sorbitol biosynthesis and phloem concentrations increased in peach tree seedlings

under stress suggesting its transport (Escobar-Gutierrez et al., 1998). A similar phenomenon was observed in soybean with pinitol under high temperature stress (Guo and Oosterhuis, 1995). Hu et al. (1997) have reported mannitol-boron complexes in the phloem sap of celery. Boron mobility is thought to increase by forming such complexes with polyols. The presence of a mannitol transporter (*AgMaT1*) in celery phloem was reported recently by Noiraud et al. (2001) but the mechanisms involved in phloem loading and unloading are yet to be fully characterized. Mannitol dehydrogenase (MTD) is involved in the catabolism of mannitol in higher plants converting mannitol to mannose. MTD in higher plants is a 1-oxidoreductase which differs from microbial MTD (2-oxidoreductase) which converts mannitol back to M1P (Zamski et al., 2001). The activity of MTD is known to be suppressed under stress allowing the accumulation of mannitol, which can act as an osmoprotectant, antioxidant and a source of carbon (Prata et al., 1997).

Transgenic Approach: Pathway for a Second Green Revolution!

Breeding for water deficit stress takes a long time and yet has given limited success because the response to water deficit stress is a quantitative trait influenced by several genes whose individual effects are difficult to identify in pedigrees (Flowers et al., 2000). Besides, the fear of introducing undesirable genes into the breeding program is ever present. Transgenic approaches offer new opportunities to improve dehydration-stress tolerance in crops by incorporating genes involved in stress protection into species that lack them (Bajaj et al., 1999). Genetic engineering is also a faster and precise means of

achieving improved resistance to water stress because it avoids the transfer of unwanted chromosomal regions (Cushman and Bohnert, 2000).

A bacterial *mtlD* gene encoding for mannitol -1-phosphate dehydrogenase has been cloned into wheat (Abebe et al., 2003) for synthesis of mannitol either in the cytosol or in the chloroplasts. These transgenic wheat plants showed increased tolerance to water deficit and salinity, but the amount of mannitol accumulating in the mature leaf cells was too low to contribute significantly to osmotic adjustment in leaf tissues. Improvement in stress tolerance may have been due to maintenance of water potential gradients at the whole plant level because of a role of osmolytes in the roots (Bray, 1997). Similar studies were carried out on tobacco and Arabidopsis (Tarczynski et al., 1993; Thomas et al., 1995). Tobacco roots accumulated greater amounts of mannitol suggesting that mannitol was involved in OA, whereas Arabidopsis showed very low mannitol content. But transgenic Arabidopsis plants were able to germinate in solutions containing up to 400 mM NaCl whereas the wild types ceased to germinate at 100 mM NaCl. The protection conferred by mannitol accumulation was attributed to other osmoprotectant functions and free radical scavenging. Chaturvedi et al. (1997) reported that *mtlD* gene expression and subsequent mannitol accumulation increased osmotolerance in yeast. Photosynthetic carbon partititioning showed a shift from sucrose to mannitol in celery under stress (Everard et al., 1994) leading to lower sucrose levels under saline conditions. Similar lowering of sucrose levels was observed in transgenic tobacco accumulating trehalose under water deficit stress (Romero et al., 1997)

Though mannitol is termed a compatible solute, its accumulation in the cells affects growth and phenotype of the transgenic plants. Reported results do not always agree with each other. Transgenic tobacco plants that accumulated mannitol were 20 to 25% smaller than wild type plants in both salinity and drought experiments (Karakas et al., 1997). Karakas et al. (1997) observed that +*mtlD* tobacco plants exhibited a slower growth rate when compared to wildtype plants under no stress conditions, which contradicts the report of Tarczynski et al. (1993) that there is no difference in growth of +*mtlD* and –*mtlD* plants in the absence of stress. However *mtlD*-transformed wheat exhibited slower growth and growth abnormalities. Interestingly, the transgenic wheat plants that synthesized the highest amounts of mannitol were sterile and phenotypically abnormal (Abebe et al., 2003). In some other studies involving transgenic plants the results were as diverse and different. Fructan accumulating transgenic tobacco showed higher biomass accumulation during drought stress whereas no significant difference was seen under non-stresses conditions (Pilon-Smits et al., 1995). Trehalose accumulating transgenic plants showed improved drought tolerance but exhibited multiple phenotypic alterations such as stunted growth (Romero et al., 1997).

Oxidative stress:

Generation of ROS:

Oxygen exists in nature as a diatomic molecule (O_2) and amounts to 21% of dry air. O_2 contains two unpaired electrons in separate orbitals with parallel spins. The enormous

oxidative potential of O_2 is expressed when this spin restriction is overcome by either energy transfer or electron transfer reactions (Halliwell and Gutteridge, 1990).

Oxidative stress occurs in plants due to the accumulation of ROS in the plant tissues during stress. ROS are species of oxygen, which are more reactive than molecular oxygen. The primary ROS, superoxide is formed by one electron reduction of molecular oxygen. Further reduction of superoxide produces hydrogen peroxide (H_2O_2) and later hydroxyl radicals through Fenton and Haber-Weiss reactions. Therefore, under stress conditions, once superoxide is formed the subsequent production of H_2O_2 and hydroxyl radicals are almost inevitable (Hancock et al., 2001). ROS are continuously produced in various metabolic pathways and in different cellular compartments. The primary center of the generation of ROS in plants is the chloroplast. The photosynthetic electron transport system is the major source of ROS (Baker and Orlandi, 1999).

Singlet oxygen is formed due to the energy transfer reactions involving the chlorophyll antennae of Photosystem II (PSII) and the oxygen molecule under high light intensities. When the chlorophyll molecule remains in an excited state for a long period of time, it converts to a triplet state and subsequently transfers the excitation energy to oxygen molecule resulting in the production of singlet oxygen (Hideg et al., 2002).

Direct reduction of O_2 in Photosystem I (PS I) due to limitations in the availability of $NADP^+$ leads to the formation of the superoxide radical. High light intensities lead to excess reduction of PS I so that the CO_2 fixation cannot keep pace and $NADP^+$ pools are

reduced to NADPH. Under these conditions O_2 competes with the low amount of $NADP^+$ for electrons from PS I leading to free radical formation (Allen, 1995). Kaiser (1979) reported that when CO_2 fixation is limited due to environmental stress conditions, PS I reduction and ROS production can occur even under moderate light intensities. The oxygenation reaction of rubisco and the photorespiratory pathway results in the generation of H_2O_2 in the peroxisomes. H_2O_2 is also generated in the cells due to the dismutation of the superoxide radical by the superoxide dismutase (SOD) enzyme family. Further reduction of H_2O_2 in the presence of transition metal ions leads to the formation of the hydroxyl radical. The hydroxyl radical is the most reactive of all the ROS and is thought to react immediately at the site of its formation with little selectivity (Samuni et al., 1983). Under physiological conditions the estimated mean life time and the mean diffusion distance of the hydroxyl radical are 10^{-7} s, and 4.5 nm respectively (Asada, 1994). Other sources of ROS in plants include NADPH oxidases, cell wall bound peroxidases, amine oxidases of the apoplast and respiration (Mittler, 2002).

Role of ROS:

The dual role played by ROS in plants has become obvious in recent years: either exacerbate damage during stress or signaling the activation of defense responses (Dat et al., 2000). The ROS generating systems like NADPH oxidases, cell wall bound peroxidases and amine oxidases are known to play a central role in the activation of defense mechanisms against insects and pathogens (Grant and Loake, 2000). ROS are also implicated to play a vital role in lignification of plant cell walls and induction of senescence (Apel and Hirt, 2004). O_2 also plays an important role as an alternative

electron acceptor during photosynthesis, thus protecting the photosynthetic machinery from photoinhibition and inactivation. Takahashi and Asada (1988) showed the presence of a 'pseudocyclic' electron transport where the superoxide radical formed when oxygen accepts an electron from PSI, donates the electron to components of cytochrome complex or plastocyanin electron carriers.

However, when high amounts of ROS are generated under adverse environmental conditions, unrestricted oxidation of various cellular components can lead to the destruction of the cell (Dat et al., 2000; Asada, 1999). ROS induced cell damage can result from membrane lipid peroxidation (Selote et al., 2004; Zhao et al., 2005), protein oxidation (Stadman and Levine, 2003; Cervantes-Cervantes et al., 2005), enzyme inhibition (Shen et. al., 1997) and DNA damage (Wiseman and Halliwell, 1996; Balasubramaniam et al., 1998). Nishiyama et al. (2001) reported that ROS inhibits repair of photo damage to photosystem II (PS II) *in vivo* by inactivation of H_2O_2 scavenging enzymes in cyanobacteria.

ROS scavenging mechanisms in plants:

Cells have evolved sophisticated strategies to keep the concentrations of ROS under control. The detoxification of ROS in plants can be broadly classified into two groups: enzymatic and non-enzymatic. Enzymatic scavenging mechanisms include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT). SOD acts by catalyzing the disproportionation of superoxide radicals. Thus, the steady state levels of superoxide are reduced but the byproduct of this reaction

is H_2O_2, another species of reactive oxygen. There are three types of SODs that differ in their prosthetic metals. CuZn-SOD is the major type of SOD in plants and is present in both chloroplasts and the cytosol, Mn-SOD is localized in mitochondria and Fe-SOD, if present, is found in the stroma of the chloroplasts (Kanematsu and Asada, 1990).

APX, GPX, and CAT act in concert to detoxify H_2O_2 produced in the cells. APX is found in both chloroplasts and the cytosol. The ascorbate-glutathione cycle regenerates the ascorbate and reduced glutathione (GSH) used up in the H_2O_2 scavenging process. GPX also scavenges H_2O_2 but uses GSH as the reducing agent. GPX activity is localized to the cytosol whereas CAT is found mainly in the peroxisomes. CAT catalyses the conversion of H_2O_2 to water in the peroxisomes.

In the presence of methyl viologen PS I produces superoxide. Overexpression of a pea chloroplast SOD in tobacco increased the resistance to methyl viologen induced membrane damage (Allen, 1995). Similar results were observed in Arabidopsis (Wang et al., 2004). The levels of ROS scavenging enzymes in the cell depend on the levels of respective ROS produced, and are tightly controlled. Studies involving transgenic plants where one or more of the above enzymes were overexpressed/ suppressed showed that the scavenging mechanisms are compensatory.

Apart from ascorbate and glutathione, which are involved in scavenging with APX and GPX, several non-enzymatic components to detoxify the ROS exist. Carotenoids (Young, 1991), tocopherol (Munne-Bosch, 2005), flavonoids, phenolics and terpenoids

(Grassmann et al., 2002), and sugars and sugar alcohols (Shen et al., 1997) are known to play an important role in ROS scavenging. Overexpression of a single component of the antioxidant system has given mixed results so far with some studies showing increased tolerance and some reporting increased damage in the presence of oxidative stress (Allen, 1995).

Role of sugar alcohols in ROS scavenging:

Besides being osmolytes, compatible solutes are suggested to have other protective functions. *In vitro* studies have shown that accumulation of compatible solutes can stabilize membranes and protect enzymes against some forms of chemical denaturation (Yancey et al., 1982). Smirnoff and Cumbes (1989) evaluated the hydroxyl radical scavenging capacity of compatible solutes and confirmed that sorbitol, mannitol and myo-inositol were effective in free radical scavenging. ROS are toxic at higher concentrations and have the potential to damage membrane lipids (lipid peroxidation), proteins (proteolysis and fragmentation) and DNA (deletions and mutations) (Halliwell and Gutteridge, 1990; Asada, 1994; McKersie and Leshem, 1994).

Several studies on accumulating polyols in plant cells point to their role as scavengers of ROS and stabilizers of membranes and proteins (Galinski, 1993; Papageorgieu and Murata, 1995; Bohnert and Jensen, 1996). Mannitol is known to possess free radical scavenging properties and was reported to scavenge hydroxyl radicals *in vitro* but the products of the reactions between mannitol and hydroxyl radical are still unknown (Franzini, 1994).

The most susceptible targets of ROS are biomembranes and subcellular organelles due to their content of polyunsaturated fatty acids in membrane phospholipids and galactolipids in the thylakoid membranes. As a consequence of lipid peroxidation, membrane fluidity may be impaired, activity of membrane proteins affected, and finally the membrane will be completely disrupted and ion homeostasis lost (Grassmann et al., 2002). To avoid possible damage to plant tissues the ROS should be kept under control continuously at the cellular level. Scavenging of the excess ROS produced in the cells by engineering synthesis of compatible solutes in the cells have given some promising results. Targeting mannitol biosynthesis to chloroplasts in transgenic tobacco resulted in accumulation of mannitol under stress and increased tolerance to methyl viologen (MV) induced oxidative stress (Shen et al., 1997). The increased tolerance was due to the protection of dark reaction enzymes by mannitol. Shen et al. (1997) suggested that the stress-protective role of mannitol might be to shield inactivation of thiol-regulated enzymes like phosphoribulokinase, thioredoxin, ferrodoxin and glutathione by hydroxyl radicals. Mannitol biosynthesis is thought to be an important nonenzymatic pathway to scavenge excess free radicals.

ROS formation and regulation is a complex cellular event and oxidative damage is often associated with plant stress. Abiotic stress conditions are exacerbated by the effect of ROS accumulation (Foyer et al., 1997; Smirnoff, 1993). Drought stress results in inhibition of photosynthesis, thus leading to production of ROS (Smirnoff, 1998). Several studies have reported induction of oxidative stress during water deficient conditions (Sgherri et al., 1995; Loggini et al., 1999; Boo and Jung, 1999). Lipid peroxidation, a

commonly used indicator of oxidative stress increases in various tissues during drought (Moran et al., 1994; Gogorcema et al., 1995; Iturbe-Ormaetxe et al., 1998). Most of the above damage to cellular machinery is attributed to the activity of the hydroxyl radical. Membrane systems of the cells suffer extensive damage through the process of lipid peroxidation. Lipid peroxidation occurs due to the hydrogen abstraction reaction from the methylene ($-CH_2-$) group resulting in formation of the carbon radical. The reaction of the carbon radical with oxygen gives rise to a peroxy radical, which in turn can attack a lipid molecule and abstract a hydrogen atom thereby starting a chain reaction. Studies conducted *in vitro* have shown that lipid peroxidation increases in the presence of iron and copper salts suggesting the role of the hydroxyl radical.

Molecular Characterization of the *mtlD* Gene:

Estimation of transgene copy number:

Development of transgenic lines with genes of economic importance opens new possibilities for their utilization. Plant breeders can use the transgenic material to move the gene of interest to commercial varieties. For this purpose, the transgene must be integrated into the host genome and must be inherited in a stable manner. Direct DNA delivery methods like particle bombardment, often result in complex patterns of transgene integration along with multiple copy number (Kohli et al., 1998; Srivastava et al., 1996) affecting the inheritance and the expression of the transgene. Less than 20% of the transgenic events generated by direct delivery methods display low copy number. Transgenic plants with multiple copy number are more likely to exhibit transgene

silencing (Cluster et al., 1996; Iyer et al., 2000). Similar observations were made by Bourden et al. (2002) wherein the high luciferase activity of the transgene was not maintained in subsequent generations of wheat. The site of integration in the host genome (Iglesias et al., 1997) and the configuration (copy number and arrangement) of the integrated transgene contribute to silencing. Configuration of the transgene results in silencing at the transcriptional (Ye and Singer, 1996) and post-transcriptional level (Elmayan and Vaucheret, 1996). Transgenic pathogenesis-related (PR) proteins introduced by particle bombardment showed expression in T_0 but were silenced in T_1 and T_2 generations (Anand et al., 2003). The suitability of transgenic material for successful breeding programs can be evaluated by estimating the transgene copy number as a first step.

Southern blot analysis is the traditional method for copy number estimation. Southern blot experiments done on the T_0 lines of wheat in our experiments have shown copy numbers of *mtlD* and *bar* genes ranging from five to more than ten per haploid genome (Abebe et al., 2003). In recent years, quantitative real-time PCR (qRT-PCR) has emerged as a robust and accurate method for estimating transgene copy number (Ingham et al., 2001; Schmidt and Parrot, 2001; Song et al., 2002). qRT-PCR relies on the ability to progressively monitor fluorescence emitted from specific ds-DNA binding dyes or fluorophore labeled probes that hybridize with target sequences during the exponential phase of the PCR reaction (Chiang et al., 1996). Traditional PCR depends on end point quantitation which has certain limitations like reagent limitation at later stages, inhibitors, pyrophosphate accumulation, and reduced activity of *Taq* polymerase. The above

limitations are overcome in the qRT-PCR by collecting data during the exponential phase (Ginzinger, 2002). The number of cycles required to generate enough fluorescent signal to cross the threshold level is called the threshold cycle (Ct). These Ct values are proportional to the amount of starting template and serve as the basis for calculating copy number.

Relative quantification and absolute quantification are the two methods available for copy number estimation. The absolute method utilizes known concentrations of the plasmid containing the transgene and its dilution series to come up with the standard curve. This standard curve is used to estimate the copy numbers in the samples (Schmidt and Parrot, 2001). The relative quantification method utilizes an endogenous reference gene whose copy number is known to estimate the transgene copy number (Ingham et al., 2001, Li et al., 2004, Weng et al., 2004). The relative method is more popular because of the availability of well characterized reference genes as well as being less prone to errors than the standard curve method (Ginzinger, 2002). The copy numbers estimated by qRT-PCR have shown very high correlation with the results of Southern blot results and hence stands validated (Ingham et al., 2001; Song et al., 2002; Li et al., 2004).

Quantification of transgenic expression:

Northern blots and RNA protection assays have been used for quantification of RNA expression but are highly labor intensive. Quantitative RT-PCR has recently emerged as the technique of choice for studying mRNA expression levels (Ginzinger, 2002; Wong and Medrano, 2005). Availability of good fluorescent detection chemistries and

sophisticated instruments that allow us to follow the reaction in real time has made the quantification more precise, high throughput and less labor intensive. The expression level of the transgene may vary within different transgenic plant lines due to position effect and copy number (Bajaj et al., 1999). Since transgene silencing is commonly noticed in plants with high copy number, it is necessary to monitor the expression of the transgene beyond the T_0 generation.

Relative quantification of transgene expression utilizes an endogenous reference gene (housekeeping gene) for normalization of gene expression. It is necessary to select a reference gene that is constitutively expressed and does not fluctuate under stress conditions for normalizing the data. There are several genes available for use as reference genes such as β-, γ-actins, albumin, α-,β-tubulins, cyclophilin, G3PDH, L32 ribisomal protein etc. (Thellin et al., 1999). Recent studies have suggested that each of the reference genes mentioned above may vary in expression levels under different conditions (Thellin et al., 1999). So, it is necessary to screen several housekeeping genes and select the one which shows the least variation or use the geometric mean of several housekeeping genes for normalization (Vandesompele et al., 2002).

Objectives:

Transgenic wheat lines with the *mtlD* gene encoding for mannitol-1-phosphate dehydrogenase were evaluated at the biochemical, physiological, and molecular levels for responses to water deficit stress. Several protective responses are thought to be provided

by mannitol such as osmoregulation, osmoprotection and ROS scavenging at the cellular, tissue and whole plant levels. Wheat transformed with the *bar* selective marker gene (-*mtlD*) and wild type bobwhite were used as controls during the experiment. The T_3 and T_4 progenies from four transformation events were used for this research.

The specific objectives were:

1. To evaluate the physiological effects of mannitol accumulation in transgenic wheat under water deficit stress and non-stress conditions.
2. To estimate the levels of sugar and sugar alcohol accumulation in leaf tissue of the transgenic plants in response to water deficit stress.
3. To study the role of mannitol in oxidative stress protection of the transgenic wheat under water deficit stress.
4. To quantify *mtlD* gene expression and copy number in transgenic wheat using quantitative real-time PCR (qRT-PCR).

CHAPTER III

METHODOLOGY

Plant materials:

Seeds were surface sterilized using a modified procedure described previously by Speakman and Kruger (1983). Seeds were surface sterilized in 1% silver nitrate (Sigma, St. Louis) in a sonicator for 30 s. The seeds were immediately transferred to deionized water and rinsed thoroughly for 5 min by replacing the water at least three times. Seeds were placed on autoclaved filter paper saturated with 100 mg Kg^{-1} piperacillin in 1% DMSO solution. The petri dishes were wrapped in aluminium foil to keep the seeds in darkness. The seeds were allowed to imbibe the solution at room temperature for 24 h. Seeds were then moved to 4°C for 48 h to overcome seed dormancy. The seeds were next soaked in 0.4 % Terrachlor 75% WP (Pentachloronitrobenzene) (Uni Royal Chemical Company, CT, USA) and allowed to germinate. After 4 d, 5-8 cm long seedlings were planted in conetainers in the growth chamber. The seedlings were screened for the transgene by PCR at the two to three leaf stage and positive plants were transplanted into pots approximately 2 wk later.

Polymerase chain reaction (PCR):

Multiplex PCR analysis was performed on the leaf tissue when the plants were at the two to three leaf stage to confirm the presence of the bar and *mtlD* genes in the transgenic wheat plants. The DNA extraction and PCR analysis for the screening of plants was done using the REDExtract-N-Amp plant PCR kit (Sigma, St. Louis). DNA was extracted from ~50 mg leaf tissue by incubation in 100 µl of extraction buffer for 10 min at 95°C

and adding an equal quantity of dilution buffer. This extract was used as template for PCR reactions. The sequences for the *mtlD* primers were 5'-CGG GTA TCC AAC TGA CGT TT-3'; 5'-CCG TGT TCA GGG TGA AGA GT-3', and the sequence for the bar gene primers were 5'-CAT CGA GAC AAG CAC GGT CAA CTT C-3'; 5'-CTC TTG AAG CCC TGT GCC TCC AG-3'. The sizes of the amplified fragments were 600 bp for *mtlD* and 300 bp for the *bar* gene. The master mix for PCR was provided with the Sigma kit. The initial denaturation step for 3 min at 95°C was followed by repeated cycles of 95°C for 1 min, 52°C for 1 min and 72°C for 1 min. A final extension was done at 72°C for 5 min. The final product was separated electrophoretically on a 1.2% agarose gel.

Southern blot analysis:

Genomic DNA was extracted from leaf tissue using DNeasy plant mini kit (Qiagen Inc., Valencia, CA). Ten micrograms of DNA for each sample was digested with EcoRI, and BamHI in separate centrifuge tubes overnight at 37°C. Later the digested DNA was precipitated in phenol:chloroform and resuspended in 20 µl sterile water. The digested and concentrated DNA samples were loaded into a 0.8% agarose gel and electrophoresed for 14 h at 30 volts. The DNA was depurinated by incubating the gel in 0.25 M HCl for 5 min and rinsed in sterile water. The gel was placed in denaturing buffer on a rotary shaker for 30 min and rinsed in sterile water. Then the gel was placed in neutralization solution for 15 min. The DNA was transferred from the gel to HybondN+ nylon membrane (Amersham LifeSciences, Arlington Heights, IL) by upward capillary transfer in 20X SSC by leaving it overnight. The membrane was briefly rinsed in 2X SSC to remove any agarose sticking to it and the DNA was fixed to the membrane by UV

crosslinking. The probe was a 600 bp long PCR product of the *mtlD* gene labeled with $\alpha[^{32}P]$-dCTP using the RediPrime labeling systen (Invitrogen, Carlsbad, CA). The probe was hybridized in a cylindrical glass container in a rotisserie overnight. The membrane was rinsed in different concentrations of 2X SSC/SDS solutions to the required level of stringency to remove any non-specific radioactivity. The membrane was autoradiographed for one to two days on a photographic film at –80°C.

Objective 1: To evaluate the physiological effects of mannitol accumulation in transgenic wheat under water deficit stress and nonstress conditions.

A thorough study of several physiological parameters thought to be influenced by mannitol accumulation in the transgenic plants was carried out. Water deficit stress was imposed on the experimental plants with some modifications to the method used in the study by Abebe et al. (2003). Time domain reflectometry (TDR) was used to assess the volumetric water content (VWC) of the soil. Plants were grown in pots in a greenhouse at 22°C/ 18°C, day night temperatures with a 14 hour photoperiod. The pots were 22.9x21.6x17.8 cm in size with a capacity of 7.8 L. The pots were filled with Redi-earth plug and seedling mix (Sun-Gro Horticulture, Bellevue, WA) and fertilized with Peters Professional 20:20:20 water soluble fertilizer and Osmocote 14:14:14 slow release fertilizer (The Scotts Company, Marysville, OH). Peters Professional 20:20:20 water soluble fertilizer was used during the first 2 wk after transplanting and discontinued a few days after application of Osmocote 14:14:14 slow release fertilizer. According to the manufacturer the slow release fertilizer will supply nutrients to the plants for 3 mo.

Water Potential Parameters:

Water potential is widely accepted as a fundamental measure of a plant's water status and a sensitive indicator of plant water stress. Solute potential and turgor potential are the two major components of water potential. Water potentials and solute potentials were measured in the leaf tissues using the HR-33T dew point microvoltmeter and C-30 psychrometers (Wescor Inc., Logan, UT). Water potentials and osmotic potentials were measured using the dew point mode because it is less affected by changes in ambient temperatures than the psychrometric mode. Standard curves were developed for all the psychrometers using different concentrations of NaCl. Water potentials for NaCl solutions ranging from 0.1-0.6 molal concentrations at 30°C (Lang et al., 1967) were used for the standard curves.

Water potentials and osmotic potentials were measured at two different time points, i.e. 15 days and 30 days after the imposition of water stress. Measurements were taken from the base and the middle portion of the youngest leaf other than the flag leaf. A leaf fragment approximately 5 cm long was loaded into the psychrometer chamber and allowed to equilibrate at 30°C for 1.5 h. Then, the psychrometers were connected to the HR33-T dew point microvoltmeter and the voltage generated due to the cooling of the thermocouple was measured. For determining the osmotic potentials the psychrometers were frozen in liquid nitrogen for 15 min and thawed to room temperature before measuring the voltage again. Freezing the samples in liquid nitrogen results in the breakdown of cellular membranes causing loss of turgor and therefore allowing us to

determine the osmotic potentials. The microvolt measurements were converted to water potentials and osmotic potentials (MPa) using the standard curves and the equation

$$\mathbf{Y = m}x\mathbf{+b}$$

where Y is the water/osmotic potential in MPa, m is the slope, b is the Y-intercept and x is the microvolt values measured on the microvoltmeter.

Osmotic adjustment (OA):

OA involves the net accumulation of solutes in a cell measured at full turgidity in response to a fall in the water potential of the cell's environment. As a consequence of this net accumulation, the osmotic potential of the cell is lowered, which attracts water into the cell and tends to maintain turgor (Blum et al., 1996). OA was calculated as the difference in measured osmotic potential at full turgor between nonstressed and stressed leaves after rehydration (Blum, 1989; Chandrababu et al., 1999). Leaf fragments of approximately 5 cm were floated on distilled water in petri dishes for 2 h immediately after harvest. Later the leaf tissue was blotted dry, loaded into the psychrometer chamber and frozen in liquid nitrogen for 15 min to breakdown cellular membranes. The leaf chamber was allowed to equilibrate for 1.5 h at 30°C before the microvolt measurements were taken with an HR33-T dewpoint microvoltmeter. The osmotic potentials were calculated from the microvolt measurements using the standard curves for the individual psychrometer. Data were collected from three replications for each treatment for the water potentials, osmotic potentials and OA.

Relative water content:

Relative water content (RWC) of the leaf was measured following the procedure modified from Sharp et al. (1990). Approximately 2 cm long leaf segments were used for this experiment. Leaf tissue was transported on ice in sealed plastic bags to the laboratory and the fresh weights taken immediately. Then the leaf tissue was transferred to centrifuge tubes filled with cold deionized water and placed on ice for 4 h. Later the leaf tissue was removed, blotted with tissue paper to remove excess water from the surface and the turgid fresh weight was taken. The leaf tissue was next placed in different centrifuge tubes and dried in a hot air oven at 70°C for 48 hours and the dry weight taken. The RWC was calculated by using the formula:

RWC= (FW-DW)/(TFW-DW)*100

The RWC is expressed as a percentage at a given time of the water content at full turgor. RWC data was collected from three replications for each treatment and collected along with OA samples.

Gas exchange measurements**:**

CO_2 assimilation rate or net photosynthesis (A), stomatal conductance and transpiration rate were measured using a LI-6400 portable photosynthesis system (LI-COR Inc, Lincoln, NE). The LI-6400 is an open system, wherein the CO_2 concentration, humidity and temperatures can be conditioned. The LI-6400 mixes CO_2 with the air going into the chamber so that it is at a set CO_2 concentration and measures the CO_2 coming out of the

chamber and then calculates the A from the difference in the two CO_2 concentrations and the air flow rate. The CO_2 concentrations in the reference and the sample chambers are measured by infrared gas analyzers present in the sensor head. A humidity sensor in the sensor head is used to measure the concentration of H_2O. Light intensity of 1000 μmol m^{-1} s^{-1}, 380 μmol mol^{-1} of CO_2, 50% relative humidity and 22°C temperature were maintained in the chamber.

Net photosynthesis (A), transpiration rate, and stomatal conductance were measured at weekly intervals after the imposition of water stress. Measurements were taken at noon three times (7, 14, 21 days after the beginning of stress) in the T_3 and 4 times (7, 14, 21, 28 days after the beginning of stress) in the T_4 generation. Water stress was imposed on the plants starting from stage 5 on the Feeke's scale. Data shown is from four replications for each treatment.

Chlorophyll fluorescence:

Chlorophyll fluorescence at room temperature is almost exclusively emitted by PS II. As an indicator of stress, fluorescence measurements are appropriate because PS II is one of the most susceptible processes to stress. Furthermore, chlorophyll fluorescence can be taken as an indicator of oxidative stress as free radicals are known to cause as well as inhibit repair of photo damage to PS II (Nishiyama et al., 2000). An OS-500 modulated fluorometer (Opti-Sciences, Haverhill, MA) was used to measure the fluorescence parameters (Fv/Fm) at 14 and 28 days after stress. The leaves were dark adapted with

dark adaption cuvettes for approximately 30 min before the fluorescence was measured. Fo, Fm, Fv, and the ratio Fv/Fm were collected.

Phenotype measurements:

Height (cm) and number of effective tillers per plant were taken from all the treatments to verify the effect of mannitol accumulation on the phenotype of the transgenic plants. Fresh and dry weights of the above ground biomass were measured on the T_4 plants.

Objective 2: To estimate the levels of sugar and sugar alcohol accumulation in leaf tissue of transgenic plants in response to water deficit stress.

Sugar alcohols (mannitol, and sorbitol) and sugars (fructose, glucose, and sucrose) in the leaf tissue were determined following the method described by Adams et al. (1993). Sugar and sugar alcohol contents were estimated in leaf tissue both under stress and nonstress conditions. Two hundred milligrams of tissue were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Two volumes of ethanol/chloroform/water (12:5:3) were added to the powder and mixed thoroughly by vortexing. An equal volume of water was added and centrifuged at 10000xg for 5 min. The upper aqueous phase was transferred to a new tube and the pellet reextracted twice with water at 60°C for 30 min. Then the extracts were pooled and dried in a speedvac for approximately 4 h. The pellet was resuspended in 300 µl water and passed through a preconditioned C_{18} solid phase extraction column (Alltech Associates, Inc., IL) to remove hydrophobic substances. Later 700 µl of water was passed through the column to collect

the whole sample. The sample was then diluted five-fold for carbohydrate determination. A high performance anion exchange chromatography (HPAE) system coupled with a pulsed amperometric detector (PAD) was used for carbohydrate separation. A CarboPac PA1 ion exchange column (Dionex Corporation, Sunnyvale, CA) was employed in the experiment. Fifty microliters of the diluted sample was injected into the sample loop connected to the ion exchange column that was maintained at room temperature. Samples were separated isocratically in 40 mM degassed NaOH at a 2.0 ml min^{-1} flow rate. Authentic carbohydrate reagents (sorbitol, mannitol, glucose, fructose and sucrose) purchased from Sigma (St. Louis, MO) were used as standards. The data shown is the average of two replicates. The carbohydrate content of the unknown samples was determined based on the peak areas produced by the known concentrations of the standards.

Objective 3: To study the role of mannitol in oxidative stress protection of the transgenic wheat under water deficit stress.

Lipid peroxidation assay:

Lipid peroxidation is considered to be the most important mechanism of cellular membrane deterioration and is induced by reactive oxygen species. The level of lipid peroxidation in the leaf tissue was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid (TBA) reaction according to Dhindsa et al. (1981). Leaf sample (250 mg) was homogenized in 5 ml 0.1% trichloroacetic acid (TCA) and centrifuged at 10000x g for 5 min. One milliliter of the supernatant was aliquoted into a

fresh tube to which 4 ml of 20% TCA containing 0.5% TBA was added. The mixture was heated at 95°C for 30 min and quickly cooled on ice. The samples were centrifuged again at 10000x g for 10 min and the absorbance measured at 532 nm. Non-specific absorbance measured at 600 nm was subtracted to remove the background. The concentration of the MDA in the samples was determined using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Chemiluminescence Test for Hydroxyl Radicals:

The ABEL® antioxidant test kit (Knight Scientific Limited, Plymouth, UK) for hydroxyl radicals with pholasin was used to measure the antioxidant capacity of plant samples. Hydroxyl radicals are generated instantaneously when solution A and solution B are mixed. Pholasin, a photoprotein, reacts with reactive oxygen species to emit light (Dunstan et al., 2000; Swindle et al., 2002). Mannitol, a known scavenger of hydroxyl radicals competes with pholasin for hydroxyl radicals. Leaf extract samples prepared for total soluble sugars determination were used for measuring the antioxidant capacity. Five microliters of sample, 20 ul of the assay buffer, 50 ul of pholasin, and 100 ul of solution A were mixed in a cuvette and loaded into the luminometer. Then 20 ul of solution B was added and the luminometer read the peak luminescence (measured as relative light units) for the next 20 seconds. A no-sample control was run along with the samples to calculate the percentage inhibition of luminescence using the formula:

[(Peak, control) – (Peak, sample)] x 100/ (peak, control)

The antioxidant capacity of the sample is expressed as the percentage inhibition of luminescence of pholasin compared to the no-sample control. Mannitol standards provided along with the kit was used to derive a standard curve.

Objective 4: To quantify *mtlD* gene expression and copy number in transgenic wheat using quantitative real-time PCR (qRT-PCR).

Determination of Transgene Copy Number:

Relative quantification method was used to determine the copy number of transgenes integrated in the wheat genome of the transformed plants. Genomic DNA was extracted from the leaf tissue of transgenic plants using the DNeasy plant mini kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. One hundred milligrams of leaf tissue were used for the DNA extraction and the pellet was resuspended in 100 µl of sterile water. The quality of DNA was ascertained by running the samples on a 1.0% agarose gel and quantified using a Nanodrop spectrophotometer for use in further downstream applications.

Primers and probes for the *mtlD* and *puroindoline-b* (*Pin-b*) genes to determine transgene copy number were designed using the PrimerQuest software (Integrated DNA Technologies, Coralville, IA). The primers were obtained from Integrated DNA Technologies (Coralville, IA) and the probes from Biosearch Technologies (Novato, CA). Both the probes were labeled at the 5'- end with FAM (6-carboxy-fluorescein) as the reporter and at the 3'- end with BHQ-1 (Black hole quencher-1) as the quencher.

Unlike other quencher molecules, the BHQ does not show native fluorescence, hence eliminating background fluorescence. The sequences of the primers and probes are provided in the table below:

Target gene		Sequences (5'- 3')	Amplicon Size (bp)
mtlD	Primer1 Primer2 Probe	aaa ggc cat gtg atg aac gc tcg ctg aag gtt tct acc gt agc gtg ggt aga aga aca cgt tgg ctt tgt	152
Pin b	Primer1 Primer2 Probe	cgt gat gga gcg atg ttt ca gcg aca ttg tgg tgc tat ct tga gca tga ggt tcg gga gaa gtg ctg caa	134

IQ supermix (Bio-Rad Laboratories, Hercules, CA), an optimized PCR mastermix was used for the real-time PCR. The master mix (Cat No: 170-8860) contained 100 mM KCl, 40 mM Tris-HCl pH 8.4, 1.6 mM dNTP's, iTaq DNA polymerase (50 units/ml), 6 mM $MgCl_2$, and stabilizers.

Real-time PCR reactions were performed in 48/96 well plates using the iCycler IQ Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The *mtlD* and *puroindoline-b* genes were amplified in separate wells and all the reactions were run in triplicate. The total reaction volume was 20 µl. Ten microliters of the IQ supermix was added to each reaction along with 250 nano moles of each primer and 125 nano moles of

the probe. Since the gene of interest and the endogenous reference genes were amplified in separate wells, equal amounts of genomic DNA were added to all the wells after spectrometric quantification. Sterile water made up the rest of the reaction volume. The PCR reaction was performed as follows: 5 min at 95°C, followed by 40 cycles of 30 sec at 95°C and 1 min at 60°C that allowed completion of both the annealing and extension steps. Post-run analysis was done according to the manufacturers instructions and run on the instrument's software.

The cycle number at which the amount of amplified target gene reaches a fixed threshold is called the threshold cycle (Ct). This value is representative of the starting copy number in the original template and is used in the calculation of the number of transgene copies per genome. Amplification efficiency of the reaction is the most important consideration when using the relative method of quantification. The amplification efficiencies of the *mtlD* and *puroindoline-b* were determined by using a two fold dilution series of genomic DNA samples and a standard curve obtained. Then the copy number of the transgene in the samples was calculated using the formula:

$$X_0/R_0 = 10^{[(Ct,x-Ix)/Sx]-[(Ct,r-Ir)/Sr]}$$

Where Ix and Ir are the intercepts of the standard curves of the gene of interest and the reference genes, Sx and Sr are the slopes of the standard curves of the gene of interest and the reference genes, and Ct,x and Ct,r are the threshold cycles of the gene of interest

and reference genes upon amplification of the samples. The copy number of the gene of interest (X_0) can be calculated when the copy number of the endogenous reference (R_0) is known (Weng et al., 2004; Livak and Schmittgen, 2001).

The *puroindoline-b* gene, which was used as the endogenous reference in this experiment encodes for a protein by the same name affecting grain softness in wheat (Giroux and Morris, 1998). *Puroindoline-b* is located on chromosome 5D, and exists as two copies in the hexaploid wheat genome (Gautier et al., 1994; Tranquilli et al., 1999) and was previously used to calculate transgene copy number in wheat (Li et al., 2004).

Transgene expression profiling:

Real-Time RT-PCR has emerged as a precise and robust molecular biology technique to quantify mRNA expression levels (Ginzinger, 2002). A PCR master mix containing SYBR Green1 (Molecular Probes, Carlsbad, CA), a dsDNA binding fluorescent dye, was used for quantification experiments. Relative quantification of transgene expression was studied using the wheat β-actin gene as the endogenous control.

Total RNA was extracted from the transgenic plants using Fenozol reagent (Active Motif, Carlsbad, CA) following the procedure suggested by the manufacturer. Three hundred milligrams of leaf tissue was used for the total RNA extraction. RNA pellets were resuspended in sterile water and stored at –80°C for further applications. RNA samples were run on a 1% agarose gel to ascertain the quality and was quantified using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmimgton, DE).

Total RNA samples were treated with DNase prior to cDNA synthesis. Deoxyribonuclease I, Amplification grade (Invitrogen) was used according to the manufacturers instructions to remove all traces of DNA contamination from the total RNA samples. SuperScript II reverse transcriptase (Invitrogen) was used for the cDNA synthesis and the procedure recommended by the manufacturer was followed. Three micrograms of total RNA was added to the 20 µl reverse transcription (RT) reaction for all samples. One microliter of RNaseOUT was added to remove RNases from the samples. cDNA samples were stored at –20°C and subsequently used in the real-time PCR.

Relative quantification of gene expression was performed with β-actin of wheat as the endogenous reference to normalize the gene expression data. Real-time PCR was performed on an iCycler IQ real-time detection system (Bio-Rad Laboratories, Hercules, CA) and the IQ SybrgreenPCR master mix supplied by the same manufacturer was used. The primers were designed using the PrimerQuest software and obtained from Integrated DNA Technologies (Coralville, IA). The *mtlD* primers were 5' -AAA GGC CAT GTG ATG AAC GC-3' and 5'-TCG CTG AAG GTT TCT ACC GT-3' and the primers used for the β-actin gene were 5'-CCT TCC ACA TGC CAT CCT TC-3' and 5'-TGC TTC TCC TTG ATG TCC CT-3'. Standard curves were obtained for both the gene of interest and the reference gene using a two fold dilution series to ascertain the amplification efficiencies. The *mtlD* and the β-actin genes were amplified in separate wells for the same samples and compared. The transgene expression was determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). One of the experimental

samples serves as the calibrator and is used to generate the relative expression levels. Melt-curve analysis was performed along with every PCR run to make sure that primer dimers and other nonspecific products are not contributing to the gene expression.

Statistical analysis:

The experiment was a completely randomized design with six wheat lines and two treatments. The proc glm procedure for one way analysis of variance in SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC) was used.

CHAPTER IV

RESULTS

Water stress imposition:

Water deficit stress was imposed on experimental plants by withholding water and measuring the volumetric water content of the potted soil with TDR. Nonstressed treatments had approximately 30% VWC or above whereas the stressed treatments were maintained around 10-12% VWC throughout the period of stress (Fig 4.1).

PCR screening for the transgene:

Multiplex PCR analysis was performed on all the plants prior to their use in stress experiments to test for the presence of the *bar* and *mtlD* genes (Fig 4.2). Plants that were positive for the *bar* and *mtlD* genes were selected for further experiments. The amplicon sizes were approximately 300 bp and 600 bp for the *bar* gene and the *mtlD* gene respectively. All plants negative for the transgenes were discarded.

Southern analysis:

Southern blot analysis was performed on the transgenic plants after PCR screening. Pooled DNA from several plants of the T_3 generation was digested using EcoR I, and BamH I restriction endonucleases (New England Biolabs, Ipswich, MA). Southern analysis confirmed the inheritance of the *mtlD* gene into the T_3 generation in all the four lines used in the experiments (Fig 4.3).

Growth parameters: Fresh weights and dry weights of plants were determined in T_4 at 30 d after stress (Table 4.1). There was no difference in either fresh weights or dry weights among all the lines in the stressed treatment. Significant differences among lines were observed in the nonstressed treatment. TA2-118 and TA5-108 recorded the highest and

lowest biomass, respectively, in the unstressed treatment. Plant height and number of effective tillers (tillers with a spike) per plant were collected at 30 d after stress (Table 4.2) in T4. TA2-118 and TA2-110 were consistently taller than other lines. There was no difference among lines in the number of tillers.

Water relations:

There was no difference in RWC among the lines under unstressed conditions in both the generations (Tables 4.3 and 4.4). In the stress treatment, RWC showed a decrease in all the lines. TA2-110 and TA2-118 showed significantly higher RWC than other lines in T_3. A similar trend continued in T_4 but the difference was not significant.

There was no difference in Ψw among different lines in nonstressed treatment in both the generations (Tables 4.5 and 4.6). Water stress resulted in decrease in Ψw in all the lines and there was no pattern among the lines. All the wheat lines adjusted osmotically at both time points. Wheat lines showed higher OA values at 15 d after stress compared to 30 d after stress.

Gas exchange parameters:

Net photosynthesis rates, transpiration rates and stomatal conductance were measured at 7, 14, 21 days in T_3 and 7, 14, 21, and 28 days in T_4 after stress imposition (Figs 4.4 to 4.9). In both the generations transgenic plants showed higher net photosynthesis rates during the last time point. Transgenic lines showed higher transpiration rates in general but this was not significant at most time points. There was no difference in stomatal conductance in both the generations under nonstressed conditions. There were significant

differences among lines in the stressed treatment in both generations but there was no pattern.

Chlorophyll fluorescence:

Fv/Fm measurements were measured at 14 and 28 d after stress imposition in both generations (Tables 4.7 and 4.8). No difference among lines or between treatments was noticed in the Fv/Fm data.

Lipid peroxidation under water deficit stress:

Higher levels of MDA were observed in the stressed plants as compared to the well watered plants (Tables 4.9 and 4.10). Data collected 30 d after stress imposition showed that stressed plants accumulated three times higher MDA than well watered plants in both T_3 and T_4 generations. Among stressed plants, transgenic lines TA5-104 and TA5-108 showed consistently lower MDA content compared to TA2-110, TA2-118 and the controls. The data were statistically significant in the T_3 but not in the T_4 generation.

Antioxidant capacity of leaf extracts:

Leaf extracts prepared for the determination of carbohydrates were used to determine the antioxidant capacity. Transgenic line TA2-110 and TA2-118 showed higher capacity to inhibit the luminescence of pholasin followed by TA5-104 and TA5-108 and controls. The inhibition of luminescence was significantly higher in the TA2 lines in both the generations (Tables 4.11 and 4.12).

Carbohydrate analysis:

Water deficit stress increased the concentrations of soluble carbohydrates in both the control and transgenic lines. Sucrose and glucose contributed to most of the increase among sugars in response to water deficit stress. Mannitol was detected in both the well watered and stressed transgenic plants at both 15 and 30 d after stress imposition except in two instances. The T_4 of TA2-118 at 15 DAS and TA5-104 at 30 DAS did not show any mannitol. Furthermore mannitol did not show a consistent increase due to water deficit stress. The increase in total soluble carbohydrates due to water stress ranged from 31-48% in T_3 and 30-40% in T_4 generations. There was no difference in the total soluble carbohydrates between 15 DAS and 30 DAS time points and between transgenic and control lines (Tables 4.13 to 4.16).

Transgene expression:

Ubi-1, the maize Ubiquitin promoter was used in the construct along with the *mtlD* gene. Since it is a constitutive promoter (Christensen and Quail, 1996), gene expression was observed in both stressed and nonstressed treatments, but stress resulted in an increased expression of the *mtlD* gene. The cytoplasmic lines (TA2-110, TA2-118) showed higher expression levels under stress than the chloroplastic lines (TA5-104, TA5-108). A general decrease in *mtlD* expression level was noticed from T_3 to T_4 generation across all the treatments. Expression was completely absent in two treatments of T_4 plants at 30 d after stress (Figs 4.10 and 4.11).

Transgene copy number:

The transgene copy number was estimated according to Weng et al. (2004). *Puroindoline-b*, was used as the endogenous reference gene because it exists as a single copy per haploid genome in wheat (Gautier et al., 1994). All the transgenic lines showed multiple copy number integration. TA2 lines had lower copy number than TA5 lines. TA2-110 (eight copies) and TA5-104 (14 copies) had the lowest and highest number of copies respectively per haploid genome (Fig 4.12).

CHAPTER V

DISCUSSION

Sugars are the major organic osmolytes that accumulate during drought stress in wheat (Munns and Weir, 1981). Several researchers have reported an increase in total sugar content in wheat due to drought stress (Kerepesi and Galiba, 2000; Abebe et al., 2003; and Nayyar and Walia, 2004), however Hanson and Hitz (1982) reported a decrease in total sugars. Our data demonstrate that water deficit stress increased total soluble sugar content in all the lines. Mannitol accumulation did not necessarily increase with stress. Abebe et al. (2003) reported that water stress increased the mannitol content by 150% in the leaves, but that data was obtained from the line TA2-115 and at only one time point (30 DAS). Furthermore, we did not see any reduction in sucrose content due to mannitol accumulation in either treatment as reported by Abebe et al. (2003). The mannitol content in the T_3 and T_4 plants in our experiment ranged from 0.10 to 0.40 µmol g^{-1} fwt which was far less than reported in tobacco (Tarczynski et al., 1993; Shen et al., 1997) and wheat (Abebe et al. 2003) where the mannitol in T_2 plants ranged from 0.6 to 2.0 µmol g^{-1} fwt. Data shows a decrease in mannitol accumulation over three generations and entire absence in two treatments in the T_4. Either there was no mannitol accumulation at that time point or it was below the detection limit of the HPAE chromatography.

Both shoot fresh weight and dry weight decreased in stressed treatments, but no difference in biomass accumulation was observed between the controls and transgenic lines as reported by Abebe et al. (2003). Mannitol accumulation did not show any effect on biomass, plant height and number of tillers under stressed conditions (Tables: 4.1 and 4.2). TA2-110 and TA2-118 were consistently taller than the controls and the TA5 lines under nonstressed conditions but the link to mannitol is not clear. Karakas et al. (1997)

reported slower growth rates in transgenic tobacco with *mtlD* than the control, but we did not notice any such differences in our experiments.

In monocot leaves the youngest cells are at the base of the leaf and the oldest cells are at the tip (Langer, 1972). Abebe et al. (2003) suggested the possibility of osmotic effects of mannitol in the growing regions of the transgenic plants during stress. Hence we used the base of the youngest leaf of the plant other than the flag leaf for measuring the Ψw and its components at 15 and 30 d after stress imposition. Ψw is a good indicator of plant health because Ψw and its components influence cell growth, photosynthesis and crop productivity (Pardossi et al., 1998). Our results did not show any difference in Ψw and OA between the controls and the transgenic lines. Under stress, plants adjusted better osmotically at earlier stage (15 DAS) than the later (30 DAS), but the amount of mannitol present in the tissue was too low to make a significant contribution to OA (Tarczynski et al., 1993; Karakas et al., 1997; Abebe et al., 2003). Though some treatments showed differences in Ψw, there was no difference in the Ψs between these treatments suggesting the differences were not due to more solute accumulation.

RWC expresses water content in percentage at a given time as related to water content at full turgor. All lines showed an expected decrease in RWC due to water stress. Transgenic lines, TA2-110, and TA2-118 showed higher RWC than other lines in T_3 and T_4, but the difference was significant in T_3 only. The above lines were taller than the rest of the lines and showed consistently greater biomass accumulation even though the difference was not significant on a dry weight basis.

Water is one of the most important limiting factors of photosynthesis in higher plants (Nilsen and Orcutt, 1996) and prolonged water deficit affects virtually all metabolic processes including the plant's capacity to fix carbon. Our data shows that the transgenic plants have higher net photosynthesis rates than the controls. Though the differences were not significant throughout the stress period, the controls had lower net photosynthesis rates at the last time point in both the generations. The initial decrease in photosynthesis can be attributed to stomatal closure but continued stress reduces photosynthesis due to nonstomatal effects (Reddy et al., 2004). The nonstomatal effects include loss of rubisco activity (Medrano et al., 1997), RUBP regeneration (Lawlor, 2002) and loss of activity of thiol regulated enzymes of the Calvin cycle like phosphoribulokinase (PRK) (Shen et al., 1997). Presence of mannitol in the chloroplasts during stress in tobacco prevented the loss of activity of PRK (Shen et al., 1997). The higher net photosynthesis rates observed in the transgenic plants may be due to the presence of mannitol in the cytosol and chloroplasts protecting enzyme activities and stabilizing macromolecules (Stoop et al., 1996; Shen et al., 1997). Water stress reduced the stomatal conductance and transpiration rate in all the lines. Stomatal conductance varied among the lines during stress but the differences were not significant. Transpiration rates followed a similar trend as net photosynthesis with the transgenic plants showing higher rates even as stress progressed. The difference was significant in T_4 at later stages.

Though prolonged water deficit stress inhibits photosynthesis and other metabolic processes, it did not have any influence on Fv/Fm. No change in the dark adapted

chlorophyll fluorescence due to water stress suggests that the maximum photochemistry of photosystem II is very resistant to drought (Cornic and Massacci, 1996) especially under low light intensities (~500 µmol m^{-2} s^{-1}) that prevailed in the greenhouse. Measuring Fv/Fm under high light intensities (~1500 µmol m^{-2} s^{-1} or above) and water deficit stress can help to understand the role of mannitol accumulation in the protection of the electron transport machinery.

ROS are formed in plants as a response to environmental stimuli and as byproducts of several metabolic pathways. However under stress conditions their formation is exacerbated resulting in oxidative stress (Mundree et al., 2002). Among compatible solutes mannitol, sorbitol, and inositol were found to be effective in scavenging hydroxyl radicals (Smirnoff and Cumbes, 1989). Formation of superoxide and H_2O_2 will finally lead to hydroxyl radical production by the Fenton reaction or the Haber-Weiss reaction in the presence of free metal ions (Hancock et al., 2001). The hydroxyl radical is the most reactive of all ROS (Halliwell and Gutteridge, 1990). In our experiment hydroxyl radicals were generated *in vitro* and their reaction with a photoprotein (Pholasin) was measured in the presence of leaf extracts as percentage inhibition of luminescence. Transgenic plants showed greater inhibition of luminescence than controls and the TA2 lines predictably showed the greatest inhibition because of their higher mannitol contents in the leaf extracts. The leaf extracts used in this experiment were prepared for carbohydrate analysis by HPAE chromatography and hence can be assumed to be free of most compounds that react with hydroxyl radicals other than the carbohydrates. There was a significant difference between the transgenic and control plants under both stressed and

nonstressed treatments. The *in vitro* radical generation system was tested by using mannitol standards and developing a standard curve. The results Tables: 4.9, 4.10, 4.11 and 4.12) demonstrate that the presence of mannitol in the cells in fact reduce the adverse effect of hydroxyl radicals. Along with mannitol, all other polyols and sugars are known to react with hydroxyl radicals though the reaction rate constants differ (Buxton et al. 1988, Smirnoff and Cumbes, 1989). So, it can be assumed that the additional amount of mannitol acts as an added advantage to the plants when the levels of other sugars and sugar alcohols are not affected.

Membranes in the cells are the most susceptible parts to damage by hydroxyl radicals due to the presence of large amounts of poly unsaturated fatty acids and the thylakoids are the major centers of ROS production (Wise, 1995; Grassman et al., 2002). MDA levels estimated in the leaf tissues after 30 days of stress showed that the transgenic lines had lower levels of MDA accumulation than the controls. The lowest levels of MDA were seen in the TA5 lines where mannitol biosynthesis was targeted to the chloroplasts. The results are in agreement with those of Shen et al. (1997) who reported that chloroplast localization of mannitol in tobacco protects the plant from oxidative stress. Samuni et al. (1983) reported that hydroxyl radicals react immediately at the site of formation with little or no selectivity. Even though the amount of mannitol in chloroplast lines was lower than that of the cytosol lines, it conferred greater protection to plants from lipid peroxidation suggesting that location is more important than amount. The reaction products of mannitol and hydroxyl radical are not known, but hydroxyl radicals react with alcohols by abstracting hydrogen and forming water (Baker and Orlandi, 1999). Hu

et al. (1997) reported that boron forms complexes with mannitol and other polyols which helps it in phloem translocation. Since the formation of hydroxyl radicals by Fenton and Haber-Weiss reactions occur in the presence of transition metal ions like Fe^{+2} (Halliwell and Gutteridge, 1990), there is also a possibility that mannitol reduces hydroxyl radical formation by binding the metal ions instead of scavenging the radical itself.

Shen et al. (1997) demonstrated that mannitol in the chloroplasts protects tobacco from oxidative damage and our results are in agreement with their findings. However, reports on lipid peroxidation due to drought stress do not seem to agree. Loggini et al. (1999) did not see any increase in lipid peroxides when wheat plants were exposed to drought stress whereas Selote et al. (2004) reported that wheat seedlings that were acclimated to drought showed less lipid peroxides than those that were not acclimated when exposed to severe drought stress. Our data shows accumulation of MDA at 30 d of stress in the leaves. However the smaller amount of MDA accumulation due to drought stress suggests less damage to cell membranes than in the case of senescence (Dhindsa et al., 1981).

An array of antioxidant enzymes and non-enzymatic compounds are activated in the plant cells to keep the ROS from causing damage (Apel and Hirt, 2004). The activity of these antioxidants depends on the ROS levels and was found to be tightly controlled and sometimes compensate for each other (Allen, 1995). A detailed study of the activity of common antioxidant enzymes like superoxide dismutase, ascorbate peroxidase and catalase in the transgenic plants may provide further information on the utility of mannitol as an antioxidant in the plant system.

Mannitol accumulation in the T_3 and T_4 generations measured by us was lower than in the T_2 generation reported by Abebe et al. (2003). Other researchers have reported the loss of transgene expression over time due to several reasons (Cluster et al., 1996; Iyer et al., 2000; Anand et al., 2003). Real-time reverse transcription PCR was used to study the *mtlD* gene expression. The expression was normalized using the β-actin as the reference gene with the comparative Ct method (Livak and Schmittgen, 2001). TA2 lines showed higher expression levels than the TA5 lines in both T_3 and T_4 generations, but the expression was conspicuously absent in two stressed treatments at 30 d after stress in the T_4 generation. A general decrease in the level of *mtlD* expression was observed from the T_3 to the T_4 generation. Since the maize *Ubi-1,* which is a constitutive promoter, was used in the gene constructs used in plant transformation, expression is expected in both the treatments. The total absence of gene expression in some treatments in the T_4 generation raises the possibility of transgene silencing in later generations as reported by Bourden et al. (2002) and Anand et al. (2003). Mannitol accumulation and gene expression data presented here could not be compared because the data was collected from different sets of plants.

Direct DNA delivery methods often lead to multiple transgene copy number and complex integration patterns in the host genome (Kohli et al., 1998; Srivastava et al., 1999). Southern analyses performed by Abebe (2001) revealed the presence of multiple copy numbers in the T_0 transformants genome. The result from our real time quantitative PCR experiments for transgene copy number was in agreement with his findings. In all future

experiments the expression of the transgene needs to be confirmed to ensure that the plants have *mtlD* expression.

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Table 4.1: Fresh weight and dry weight of the above ground biomass in T_4 at 30 DAS

Stress level		Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	Fresh wt (g)	42.9±1.1abc	40.6±1.2c	44.5±1.7ab	45.7±1.0a	41.0±1.3bc	40.5±1.0c
	Dry wt (g)	8.3±0.1ab	8.1±0.1b	8.6±0.2a	8.6±0.1a	8.1±0.1b	8.1±0.1b
Stressed	Fresh wt (g)	20.4±1.0a	19.5±1.5a	22.4±0.7a	22.6±1.0a	20.9±1.0a	21.8±1.3a
	Dry wt (g)	3.9±0.1a	3.9±0.1a	4.2±0.1a	4.3±0.1a	4.1±0.2a	4.2±0.2a

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are means±SE from three replications

Table 4.2: Plant height and number of tillers in T_4 at 30 DAS

Stress level		Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	Height (cm)	59.7±1.9bc	61.7±2.5abc	67.3±1.8ab	69.7±1.3a	60.0±3.2bc	57.3±2.6c
	No. of tillers	5.3±0.3a	6.0±0.6a	5.7±0.3a	5.0±0.6a	5.3±0.3a	6.0±0.6a
Stressed	Height (cm)	42.0±2.3a	41.6±1.5a	45.6±2.3a	46.7±1.5a	42.0±1.7a	42.3±1.8a
	No. of tillers	3.3±0.3a	2.7±0.3a	3.7±0.3a	3.3±0.4a	3.3±0.3a	3.7±0.3a

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are means±SE from three replications

Table 4.3: Relative water content (%) of leaf tissue in T_3. Data measured on the mid section of the same leaf from which the Ψw data were obtained.

Stress level	Days after stress	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	15	95.2±0.5a	94.7±0.1a	95.1±0.2a	95.5±0.4a	95.2±0.6a	95.6±0.3a
	30	94.6±0.3a	94.9±0.2a	94.9±0.4a	95.2±0.5a	95.1±0.6a	94.9±0.5a
Stressed	15	80.4±0.4c	81.8±0.3b	84.0±0.4a	84.3±0.4a	81.8±0.5b	81.9±0.4b
	30	79.5±0.3b	79.6±0.4b	83.1±0.2a	83.0±0.3a	80.4±0.5b	80.2±0.5b

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are means±SE from three replications

Table 4.4: Relative water content (%) of leaf tissue in T_4. Data measured on the mid section of the same leaf from which the Ψw data were obtained.

Stress level	Days after stress	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	15	94.2±0.4a	94.3±0.3a	94.2±0.5a	94.4±0.4a	93.8±1.0a	94.1±0.9a
	30	93.7±0.8a	93.6±0.9a	93.8±0.8a	93.3±0.7a	93.8±0.6a	94.0±0.7a
Stressed	15	79.5±0.8b	80.4±0.9ab	82.1±0.6a	82.4±0.4a	80.9±0.5ab	81.5±1.1ab
	30	80.2±0.7b	80.1±0.6b	81.7±0.4ab	82.3±0.5a	82.4±0.4a	82.2±0.6a

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are means±SE from three replications

Table 4.5: Water potentials (Ψw, MPa) and osmotic adjustment (OA, MPa) in T_3. Data measured from basal part of the youngest leaf.

Stress level	Days after stress	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed (Ψw)	15	-0.65±0.02a	-0.62±0.12a	-0.61±0.03a	-0.58±0.03a	-0.62±0.02a	-0.59±0.03a
	30	-0.63±0.04a	-0.61±0.03a	-0.59±0.02a	-0.58±0.02a	-0.61 ±0.02a	-0.60 ±0.03a
Stressed (Ψw)	15	-1.93±0.02ab	-1.98 ±0.04b	-1.96±0.04ab	-1.97 ±0.02b	-1.98 ±0.04b	-1.86 ±0.03a
	30	-1.88±0.01a	-1.87±0.06a	-1.87 ±0.04a	-1.89 ±0.02a	-1.89 ±0.04a	-1.81 ±0.04a
OA	15	0.48	0.50	0.51	0.49	0.46	0.51
	30	0.23	0.24	0.23	0.19	0.22	0.18

Values of Ψw are means ± SE. OA was calculated as the difference between mean values of Ψs at full turgor in control and stressed leaves. Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are from three replications

Table 4.6: Water potentials (Ψw, MPa) and osmotic adjustment (OA, MPa) in T_4. Data measured from basal part of the youngest leaf.

Stress level	Days after stress	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed (Ψw)	15	-0.58 ±0.01a	-0.61±0.02ab	-0.62±0.01ab	-0.61±0.02ab	-0.65±0.03b	0.64±0.02ab
	30	-0.58 ±0.02a	-0.60 ±0.03a	-0.62 ±0.02a	-0.59 ±0.02a	-0.66±0.03a	-0.66±0.04a
Stressed (Ψw)	15	-1.86±0.03a	-1.90 ±0.05a	-1.86 ±0.02a	-1.86 ±0.02a	-1.85 ±0.03a	-1.83 ±0.02a
	30	-1.81 ±0.03ab	-1.87 ±0.02b	-1.79 ±0.02a	-1.82±0.03ab	-1.81 0.03ab	-1.80±0.03ab
OA	15	0.52	0.49	0.48	0.53	0.50	0.48
	30	0.19	0.23	0.20	0.15	0.18	0.21

Values of Ψw are means ± SE. OA was calculated as the difference between mean values of Ψs at full turgor in control and stressed leaves. Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are from three replications

Table 4.7: Chlorophyll fluorescence (Fv/Fm) measured on the flag leaf after 30 min of dark adoption in T_3

Stress level	Days after stress	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	14 DAS	0.84±0.004	0.83±0.002	0.83±0.004	0.84±0.002	0.84±0.003	0.82±0.003
	28 DAS	0.83±0.005	0.82±0.005	0.82±0.002	0.82±0.004	0.83±0.004	0.82±0.003
Stressed	14 DAS	0.82±0.003	0.82±0.009	0.82±0.005	0.82±0.007	0.83±0.003	0.82±0.004
	28 DAS	0.82±0.013	0.81±0.010	0.82±0.001	0.81±0.006	0.82±0.005	0.83±0.007

Values are means±SE from four replications

Table 4.8: Chlorophyll fluorescence (Fv/Fm) measured on the flag leaf after 30 min of dark adoption in T_4

Stress level	Days After stress	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	14 DAS	0.82±0.005	0.83±0.003	0.83±0.006	0.84±0.007	0.83±0.003	0.83±0.006
	28 DAS	0.82±0.001	0.83±0.001	0.82±0.005	0.83±0.006	0.84±0.001	0.83±0.003
Stressed	14 DAS	0.82±0.002	0.82±0.003	0.83±0.002	0.83±0.004	0.82±0.006	0.83±0.003
	28 DAS	0.82±0.001	0.82±0.003	0.81±0.010	0.82±0.005	0.82±0.006	0.83±0.005

Values are means±SE from four replications

Table 4.9: Malondialdehyde content of the leaf tissue in T_3 at 30 DAS (nmol g^{-1} fwt)

Stress level	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	119.9 ±3.2a	120.5 ±4.8a	115.8 ±5.9a	112.6 ±5.9a	108.9 ±5.7a	112.6 ±3.8a
Stressed	321.8 ±8.6a	317.3 ±4.2a	312.0 ±5.1a	304.7 ±4.1a	279.3 ±6.7b	283.9 ±3.8b

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are from three replications

Table 4.10: Malondialdehyde content of the leaf tissue in T_4 at 30 DAS (nmol g^{-1} fwt)

Stress level	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	112.9 ± 4.5a	115.5 ± 7.0a	118.1 ± 8.1a	113.4 ± 6.5a	111.5 ± 2.3a	116.1 ± 5.7a
Stressed	324.2 ± 7.5a	318.1 ± 5.3a	321.7 ± 9.7a	317.6 ± 4.0a	302.7 ± 8.3a	305.8 ± 7.6a

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are from three replications

Table 4.11: Antioxidant capacity of leaf extracts in T_3 at 30 DAS (% inhibition of pholasin luminescence)

Stress level	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	37.1±1.6d	36.6±1.9d	44.7±0.5ab	45.7±1.1a	41.6±1.1bc	39.5±0.9cd
Stressed	38.5±1.1c	38.7±1.3c	53.1±1.1a	51.8±2.3a	43.4±1.7bc	45.9±1.0b

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are from three replications

Table 4.12: Antioxidant capacity of leaf extracts in T_4 at 30 DAS (% inhibition of pholasin luminescence)

Stress level	Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	32.4±1.3b	33.8±2.4b	40.1±1.9a	41.3±1.1a	39.4±1.5a	37.3±1.8ab
Stressed	34.5±0.9c	34.6±2.2c	50.7±0.9a	49.4±2.2a	44.5±0.9b	43.2±1.6b

Means followed by the same letter in a row are not significantly different at $p<0.05$
Data are from three replications

Table 4.13: Carbohydrate concentration in leaf tissue in T_3 at 15 DAS (µmol g^{-1} fwt)

Stress level		Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	Mannitol	0.00	0.00	0.31	0.25	0.17	0.18
	Sucrose	13.06	12.05	12.69	13.04	11.61	12.62
	TSC	23.65	22.64	23.94	22.05	23.95	24.20
Stressed	Mannitol	0.00	0.00	0.40	0.25	0.20	0.16
	Sucrose	18.68	19.09	21.00	18.29	21.31	20.69
	TSC	30.73	31.07	33.78	28.45	33.56	36.59

Table 4.14: Carbohydrate concentration in leaf tissue in T_4 at 15 DAS (µmol g^{-1} fwt)

Stress level		Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	Mannitol	0.00	0.00	0.31	0.20	0.15	0.17
	Sucrose	12.97	12.03	12.75	12.92	11.63	12.16
	TSC	23.20	21.64	23.62	22.93	23.85	23.91
Stressed	Mannitol	0.00	0.00	0.35	0.00	0.24	0.16
	Sucrose	18.56	19.30	18.39	19.45	20.20	19.13
	TSC	31.47	32.59	31.90	32.89	32.73	33.73

Table 4.15: Carbohydrate concentration in leaf tissue in T_3 at 30 DAS (µmol g^{-1} fwt)

Stress level		Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	Mannitol	0.00	0.00	0.27	0.22	0.16	0.16
	Sucrose	13.05	12.19	12.55	12.81	11.98	12.07
	TSC	22.08	22.92	23.09	22.79	23.40	23.20
Stressed	Mannitol	0.00	0.00	0.26	0.20	0.12	0.10
	Sucrose	18.55	18.87	20.10	19.24	20.69	20.43
	TSC	28.94	30.39	33.14	29.95	32.77	34.35

Table 4.16: Carbohydrate concentration in leaf tissue in T_4 at 30 DAS (µmol g^{-1} fwt)

Stress level		Bobwhite	pAHC20	TA2-110	TA2-118	TA5-104	TA5-108
Unstressed	Mannitol	0.00	0.00	0.26	0.25	0.13	0.15
	Sucrose	12.90	12.01	12.42	12.79	11.94	12.05
	TSC	22.85	22.87	24.04	22.97	23.65	23.08
Stressed	Mannitol	0.00	0.00	0.24	0.25	0.00	0.16
	Sucrose	18.50	18.09	19.12	18.88	19.55	19.80
	TSC	29.64	30.80	31.65	30.77	31.25	32.07

Fig 4.1: Water content in the experimental pots measured using Time Domain Reflectometry (Bars are ±SE, n=4)

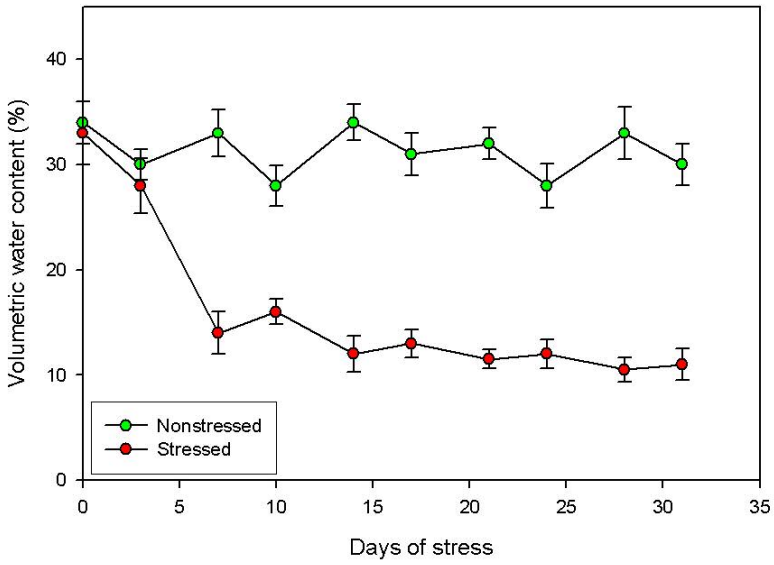


Fig 4.2: PCR screening of TA2-118 transgenic wheat line for presence of the *mtlD* (600 bp) and the*bar* (300 bp) genes

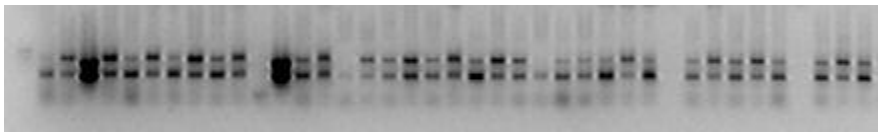


Fig 4.3: Southern blot analysis for the*mtlD* gene in T3 generation. Genomic DNA from four transgenic lines, TA2-110, TA2-118, TA5-104, and TA5-108 were hybridized with a 600 bp *mtlD* radiolabelled probe. The probe was prepared by PCR amplification of a section of the coding sequence of the *mtlD* gene

Fig 4.4: Net photosynthesis rate following imposition of water stress in T_3 (Bars are ±SE, n=4)

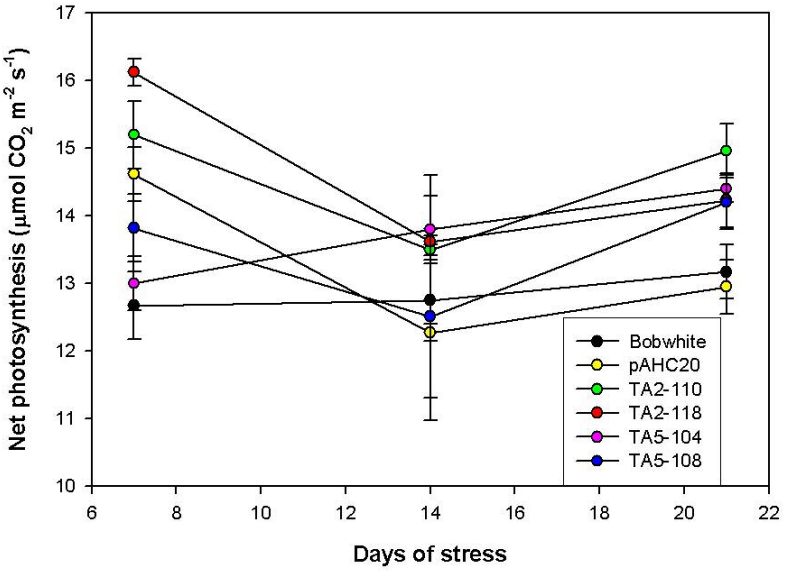


Fig 4.5: Net photosynthesis rate following imposition of water stress in T_4 (Bars are ±SE, n=4)

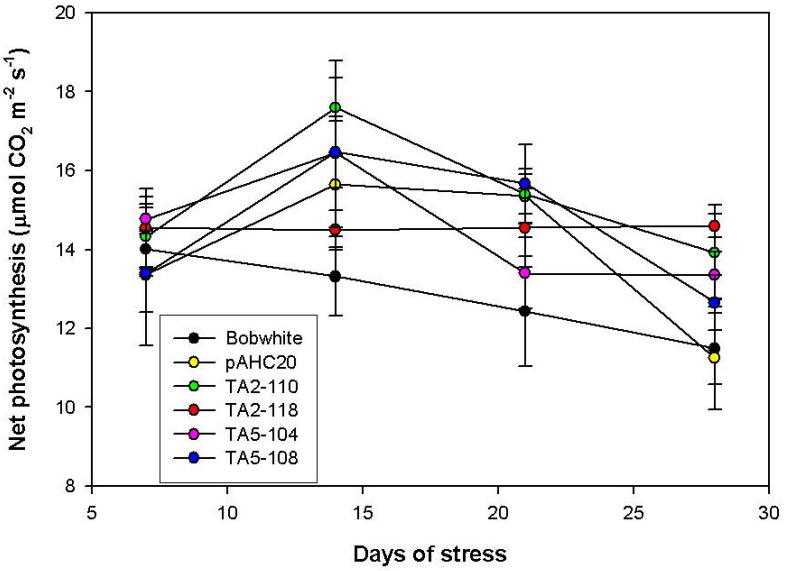


Fig 4.6: Transpiration rate following imposition of water stress in T_3 (Bars are $\pm$SE, n=4)

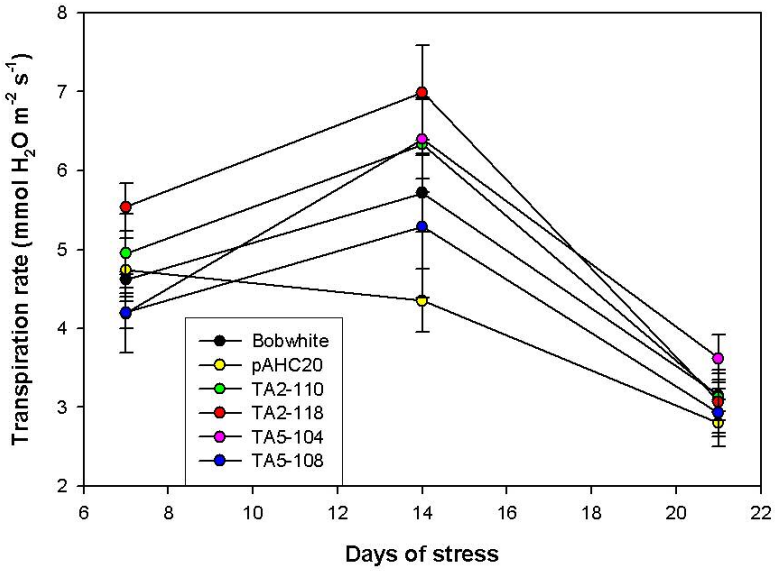


Fig 4.7: Transpiration rate following imposition of water stress in T_4 (Bars are $\pm$SE, n=4)

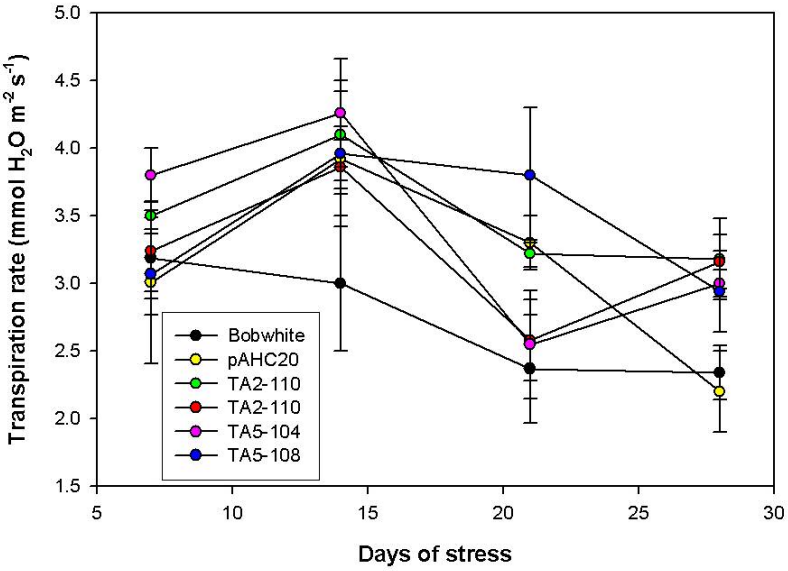


Fig 4.8: Stomatal conductance following imposition of water stress in T_3 (Bars are ±SE, n=4)

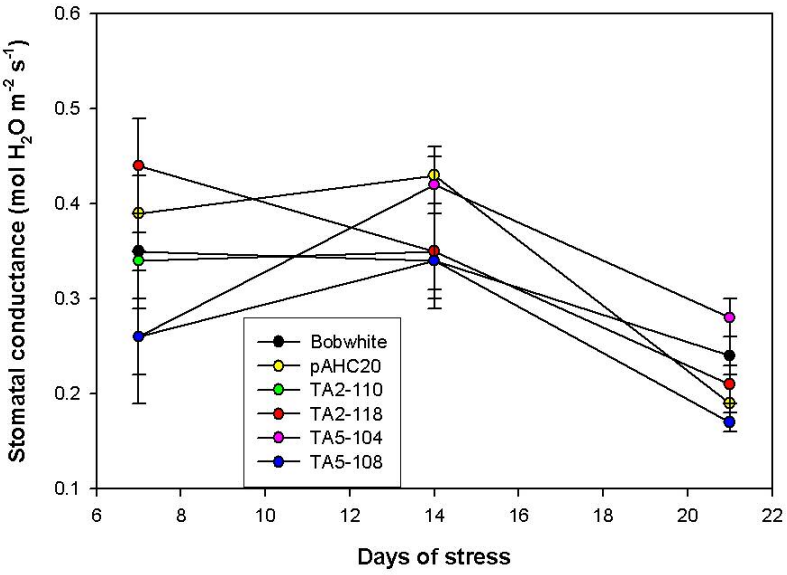


Fig 4.9: Stomatal conductance following imposition of water stress in T_4 (Bars are ±SE, n=4)

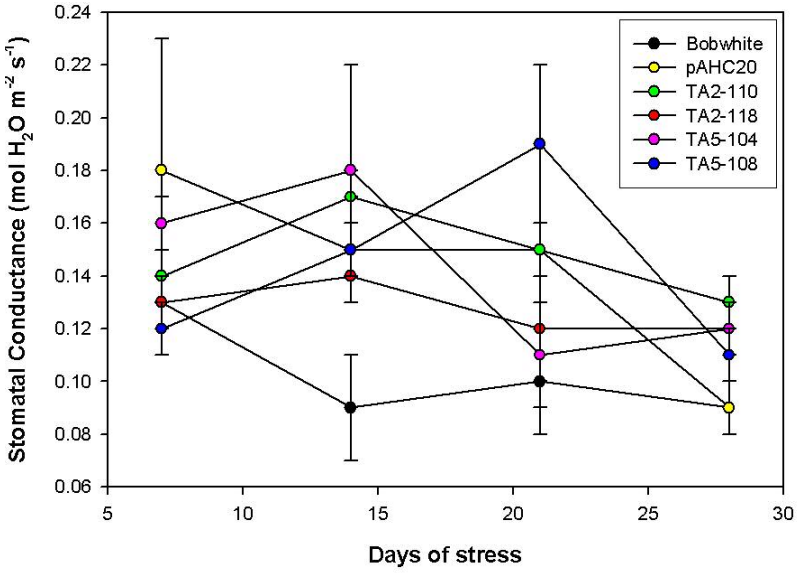
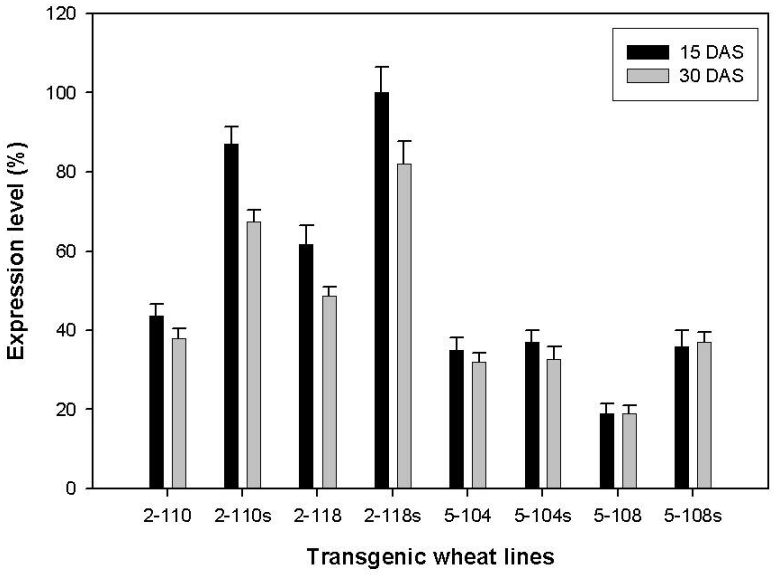
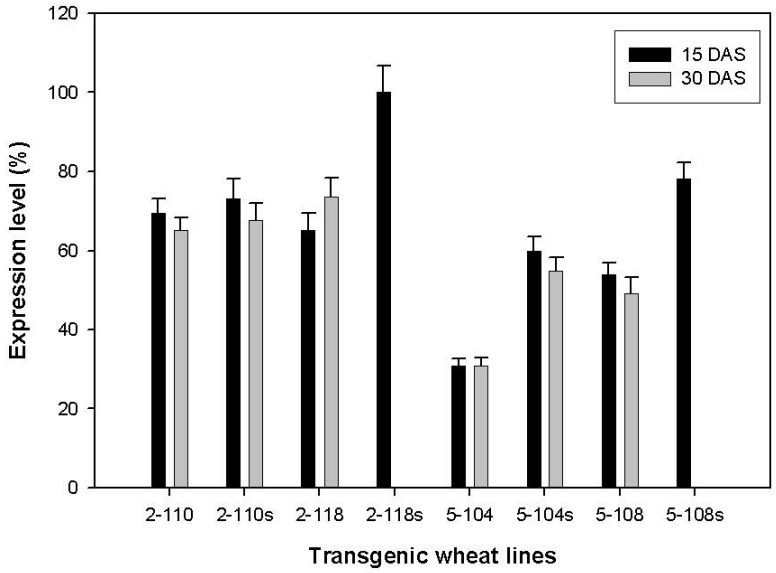


Fig 4.10: Relative expression levels of the *mtlD* gene in T_3 (2-118s at 15 DAS used as calibrator)



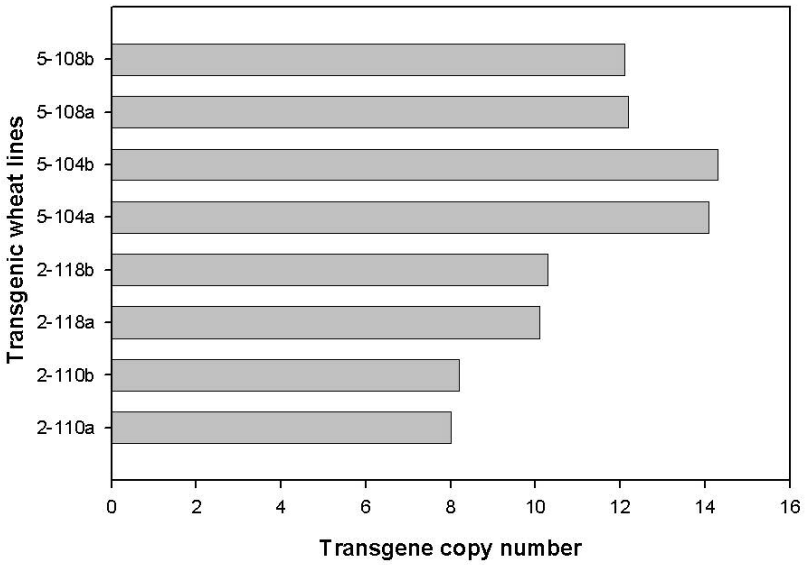
Note: 2-110, 2-118 are transgenic lines with mannitol biosynthesis in cytosol and 5-104, 5-108 are transgenic lines with mannitol biosynthesis in chloroplasts; 's' denotes the stressed treatment. Data are means±SE of three replications.

Fig 4.11: Relative expression levels of the *mtlD* gene in T_4 (2-118s at 15 DAS used as calibrator)



Note: 2-110, 2-118 are transgenic lines with mannitol biosynthesis in cytosol and 5-104, 5-108 are transgenic lines with mannitol biosynthesis in chloroplasts; 's' denotes the stressed treatment. Data are means±SE of three replications.

Fig 4.12: Number of *mtlD* copies per haploid wheat genome



Note: 2-110, 2-118 are transgenic lines with mannitol biosynthesis in cytosol and 5-104, 5-108 are transgenic lines with mannitol biosynthesis in chloroplasts. Data from two individual samples from each line is presented and denoted as 'a' and 'b'. Transgene copy number was calculated using the formula $\mathbf{X_0/R_0 = 10^{[(Ct,x-Ix)/Sx]-[(Ct,r-Ir)/Sr]}}$

VITA

Sathyanarayana Reddy Elavarthi

Candidate for the Degree of

Doctor of Philosophy

Thesis: BIOCHEMICAL, PHYSIOLOGICAL, AND MOLECULAR CHARACTERIZATION OF MANNITOL ACCUMULATING TRANSGENIC WHEAT IN RESPONSE TO WATER DEFICIT STRESS

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Pages in Study: 98 Candidate for the Degree of Doctor of Philosophy

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Abstract: Water deficit stress is a major factor that affects crop productivity. Mannitol accumulation has been reported to increase tolerance of plants to water deficit stress through several mechanisms. In this study we evaluate the effect of mannitol accumulation in transgenic wheat that has a bacterial *mtlD* (mannitol -1-phosphate dehydrogenase) gene causing mannitol biosynthesis in either cytosol or chloroplasts. The objectives were to study 1. the physiological effects of mannitol accumulation, 2. the role of mannitol in oxidative stress protection, and 3. to quantify the *mtlD* expression and copy number in the transgenic plants. Five transgenic events were used in this study, TA2-110, TA2-118 (mannitol biosynthesis in cytosol), TA5-104, TA5-108 (mannitol biosynthesis in chloroplasts), and pAHC20 (with selectable marker alone) along with the wild type (*cv* Bobwhite). Mannitol accumulation was observed in all the lines and the amount ranged from 0.10-0.40 µmol g^{-1} fwt. No difference in water relations were observed between the transgenic lines and the controls. The amount of mannitol is not great enough to make a significant contribution osmotically. Net photosynthesis rate was greater in the transgenic lines compared to controls as stress progressed but no difference in chlorophyll fluorescence (Fv/Fm) was observed. Leaf extracts from the transgenic lines showed greater capacity to scavenge hydroxyl radicals than the control *in vitro*. The transgenic lines also showed less lipid peroxidation compared to the controls as a result of stress. TA5 lines in which mannitol biosynthesis is targeted to chloroplast showed the lowest level of lipid peroxidation. Mannitol accumulation, even in small quantities imparts oxidative stress protection to the transgenic plants. Transgene expression varied among the different transgenic lines and increased under stress. TA2 lines showed greater expression than the TA5 lines. Transgene expression was absent in two treatments in the T_4 generation. The absence of transgene expression may be due to transgene silencing. All the transgenic lines had multiple copies of the *mtlD* gene integrated into the genome. With eight copies TA2-110 had the least and with 14 copies TA5-104 had the most transgene copies per haploid genome.

ADVISER'S APPROVAL: Dr. Bjorn Martin